GENETICS AND SEX EXPRESSION IN ALASKAN POPULATIONS OF *SILENE ACAULIS*

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November 22, 2004

Date

GENETICS AND SEX EXPRESSION IN ALASKAN POPULATIONS OF *SILENE ACAULIS*

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

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Fairbanks, Alaska

December 2004

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ABSTRACT

Gynodioecy, the coexistence of hermaphrodites and females within plant populations, is often caused by an interaction between maternally-inherited cytoplasmic male sterility (CMS) genes and nuclear male fertility restorer genes. Previous population studies have found cytoplasmic alleles associated with femaleness. We analyzed the spatial distributions of mitochondrial and chloroplast alleles and the sexual phenotype of individuals within five Alaskan populations of *Silene acaulis*. Sex ratios were variable between two mountain ranges in this study, possibly due to differences in the frequencies of CMS genes. Clustering of mitochondrial alleles, but not sex, was found within two populations at a scale ≤ 2 m. This result may be because maternally-inherited mitochondrial genes are locally spread through seed, but nuclear restorers are spread through pollen and seed. We also investigated sex ratios and CMS genes temporally and did not find patterns of changing sex ratios or mitotypes across size classes. This does not support the theory that females and the mitotypes they carry have been selected against over time, implying that female clusters were not broken-up due to pollen limitation. Patterns of mitochondrial and chloroplast alleles suggest either non-maternal inheritance of cytoplasmic markers or multiple reversals in the evolutionary history of cytoplasmic markers.

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Matt Olson, Ph.D., for his support and assistance through all developmental stages of this study. I would also like to thank visiting scholar, Helena Storchova, Ph.D., for her help with technical procedures used in this study. I am also indebted to committee members, Christa Mulder, Ph.D., and Pat Doak, Ph.D., for their very helpful comments on how to improve this manuscript. Heather McIntyre, UAF greenhouse manager, was of great assistance during vegetative propagation of *Silene acaulis*. Former UAF graduate student, Stephen Keller, was helpful in his recommendations for study populations. I am also very thankful to my husband, Jonathan Klaas, for the program he created to perform nearest neighborhood join counts, as well as the support and joy he and my daughter, Emma, have given me. This project was made possible thanks to the Biology and Wildlife Teaching Assistantship and Summer Research Support programs.

General Introduction

Spatial segregation of sexes (SSS) within dioecious flowering plant species has been a topic of interest following Fisher's (1930) early work on sex ratio evolution. Fisher (1930) theorized that selection would favor equal ratios of males and females within dioecious plant populations (composed of both males and females), a reflection of equal investment in males and females. However, empirical studies of sex ratios within dioecious plant populations have shown both unequal sex ratios among populations as a whole as well as patchiness of sex within populations (reviewed in Bierzychudek and Eckhart 1988). Unequal sex ratios and spatial patchiness of the sexes has also been found in populations of gynodioecious species (hermaphrodites and females in populations), where it is theorized to be a consequence of the genetics of sex determination (Cugen et al. 1994, Manicacci et al. 1996, Tarayre et al. 1997, Laporte et al. 2001, and Olson and McCauley 2002).

Spatial clustering of females has been observed in several gynodioecious species including the following: *Silene acaulis* (Hermanutz and Innes 1994, Keller 2002), *Beta vulgaris maritima* (Cugen et al. 1994, Laporte et al. 2001), *Thymus vulgaris* (Manicacci et al. 1996, Tarayre et al. 1997), and *Silene vulgaris* (Olson and McCauley 2002). Spatial clustering of females in gynodioecious species may result from maternally inherited cytoplasmic male sterility (CMS), a mechanism of sex determination in some gynodioecious species (Hanson 1991). Cytoplasmic male sterility occurs when genes located in the mitochondrial genome are expressed and interfere with normal stamen development. Specific nuclear male fertility restorers can reinstate male sterility for

some CMS types, but not others. CMS genes and nuclear male fertility restorers interact in a gene-for-gene fashion so a particular combination is required to restore male fertility (Hanson 1991, Schnable and Wise 1998). Previous genetic studies of *Beta vulgaris maritima, Thymus vulgaris,* and *Silene vulgaris* have found that clusters or subpopulations are often composed of females with the same cytoplasmic type, indicating that the spread of females carrying unrestored CMS types is responsible for female clustering (Cuguen et al. 1994, Manicacci et al. 1996, Tarayre et al. 1997, Laporte et al. 2001, Olson and McCauley 2002).

Currently, it is unknown whether gynodioecy in *S. acaulis* results from CMS, although CMS is found in the congener species, *S. vulgaris* (Olson and McCauley 2002). Highly female-biased sex ratios (80 %) were found in some populations of *S. acaulis*, which suggest maternal control of male sterility (Hermanutz and Innes 1994, Morris and Doak 1998, Keller 2002). Additionally, one study found female clustering within three of six Alaskan populations (Keller 2002). One of the primary motivations of this study was to determine if female clustering (a form of SSS) and mitotype clustering were common within Alaskan populations of *S. acaulis*. We also investigated the associations between sex expression and cytoplasmic genes. An association between sex and cytoplasmic type would strongly suggest that *S. acaulis* has CMS sex determination.

However, other ecological and evolutionary mechanisms besides, or in addition to, interactions of sex determining genes can lead to SSS. Here, I review current ecological and evolutionary hypotheses for how SSS is generated in both dioecious and gynodioecious plants. The following sections describe how sexual niche partitioning,

sex-biased mortality and reproduction on different quality sites (selection), and environmental sex determination (ESD) may contribute to SSS. In addition, the last section discusses how genetics, including cytoplasmic male sterility, can shape SSS.

Sexual Niche Partitioning

Sexual niche partitioning is a theory that focuses on evolutionary mechanisms that could cause the existence and persistence of SSS. Sexual niche partitioning theory asserts that evolution would favor males and females occupying separate habitats if they compete for the same resources (Cox 1981, Bierzychudek and Eckhart 1988). SSS via sexual niche partitioning has been implicated in the Australian python (*Morelia spilota imbricata*), the three-spine stickleback (*Oaster rosteus aculeatus*), and the tropical lizard (*Anolis conspersus*) (Schoener 1967, Pearson et al. 2002, Reichman and Nosil 2004). However, to date no study has demonstrated that sexual niche partitioning is responsible for observed patterns of SSS in plants (reviewed in Bierzychudek and Eckhart 1988).

To implicate sexual niche partitioning as the causal and evolutionary mechanism for observed cases of SSS, inter-sexual competition must be clearly responsible for SSS. Thus, other factors such as differential mortality and sex biased physiological preferences must not be found, even though sex-biased physiological factors may still evolve after selection for competition has caused males and females to occupy separate habitats. In addition, an evolutionary mechanism must be demonstrated to be responsible for SSS, such as habitat selection or environmental sex determination (ESD) (Bierzychudek and Eckhart 1988). Studies that have demonstrated that there is habitat selection or ESD have

not experimentally shown reduced inter-sexual competition to be the driving force (Bierzychudek and Eckhart 1988).

Sex-biased mortality and reproduction on different quality sites (Selection)

Sexes with higher energetic requirements during reproduction may suffer higher mortality rates in poor quality environments if reproductive output cannot be adjusted to resource availability (Lloyd and Web 1977). If reproductive output can be adjusted to resource availability sex morphs may suffer from low reproductive fitness. In dioecious plant species, females are more likely to have higher reproductive costs than males because they must mature and dispense fruits, and thus more likely to have lower fitness in stressful habitats (Bierzychudek and Eckhart 1988, Laporte and Delph 1996, Delph 2003). In this case, females of gynodioecious species may be less common on stressful sites and may exhibit population structure associated with habitat type.

In gynodioecious species, if female reproductive function is equal in females and hermaphrodites, hermaphrodites incur higher reproductive costs than females (Lloyd 1976, Delph 2003). Hermaphrodites will incur higher reproductive costs than females because they must produce pollen as well as mature fruits. Due to this cost, Lloyd (1976) hypothesized that hermaphrodites of gynodioecious species may reduce female function in stressful environments, to achieve most of their reproductive fitness from pollen. If hermaphrodites can reduce female reproductive effort to become cryptic males, then we would expect population structure of the sexes to be similar to that seen in dioecious species. In effect, females may be less common on stressful sites due to lower total

fitness compared to hermaphrodites (cryptic males). However, if hermaphrodites cannot reduce female effort, we may expect to see hermaphrodites associated with less stressful environments.

Several studies have found that among dioecious plant species, males appear to occupy poor habitat more often than females. For instance, males of the dioecious *Trophis involucrata* are more frequently found in nutrient-poor patches of low total phosphorous content (Cox 1981), and males of *Distichlis spicata* are at higher densities than females in more saline patches (Freeman et al. 1976). Other studies confirm that female plants are more common in less stressful microhabitats: sites that are moister (Freeman et al. 1976), less exposed (Bertiller et al. 2002), at lower elevations (Grant and Mitton 1979), have lower conspecific densities (Krischik and Denno 1990), or have higher nutrient levels (Cox 1981, Meagher 1984). Most studies that have reported SSS have found that it is associated with habitat type and have not been able to determine if other factors, including ESD, might also be responsible for SSS.

A study of *Plantago lanceolata*, a gynodioecious species, found that females were more frequent in moist sites than in xeric sites (Krohne et al. 1980). Moist sites had maximum female frequencies of 30%, and xeric sites had maximum female frequencies of 6.5%. The authors found that females in moist sites were able to produce more seeds than females in xeric sites. Additionally, a large proportion of females in moist sites were perennial and only perennial females were able to produce enough seeds to surpass the fitness quotient of hermaphrodites of all ages. In xeric sites, very few individuals of either sex were perennial. Thus, females appear to have a lower reproductive fitness than

hermaphrodites on xeric sites due to environment-dependent reproductive costs. Environment-biased differences in female seed production suggests that selection maintains sex ratio differences among populations.

Environmental Sex Determination (ESD)

Environmental sex determination (ESD) is a condition in which environmental and physiological conditions determine the sex of an individual or its progeny. The alteration of the sex of progeny or one's self would be advantageous when environmental conditions strongly increase or decrease the fitness of one sex over the other and when environmental conditions vary in space and time (Charnov and Bull 1977). For example, it would be advantageous for a dioecious plant to become male when conditions are so stressful that females suffer higher mortality and lower reproductive fitness than males. Similar evolutionary forces may cause selection for reduced seed set in hermaphrodites of gynodioecious species (Lloyd 1976, Olson 2001).

In ESD, sex determination is influenced by outside cues from the environment, and so theory predicts that for ESD to evolve there must be some mechanism for organisms to detect changes in the environment and override or influence the genetics of sex determination (Freeman and Vitale 1985). Environmental cues may influence sex determination at any point in the life history of the organism. ESD has been observed in marine worms and mollusks, in fish, lizards, turtles, crocodilians, and plants (Bull 1983, Freeman and Vitale 1985). In dioecious plants, environmental conditions were responsible for sex change in the sequential hermaphrodite, Jack-in-the-pulpit

(Policansky 1981), and were indicated in sex change in the orchid *Catasetum viridiflavum* (Zimmerman 1991), and *Spinacia oleracea* (Vitale and Freeman 1985).

A three-year population study of Jack-in-the-pulpit (*Arisaema triphyllum*) found that sex is significantly related to age (Policansky 1981). Jack-in-the pulpit is a dioecious species that produces one spadix composed of either male or female flowers. An individual can change sex throughout its life, independent of age. Large individuals enjoy higher reproductive fitness when female while small individuals benefit most if male. The results of this study are consistent with a size-advantage model, in which physiological conditions of the plant determine sex. In turn, physiological conditions may be strongly influenced by environment.

Sex determination in the dioecious orchid, *Catasetum viridiflavum*, was correlated with light availability, which was in turn associated with substrate type (Zimmerman 1991). A two-year population study concluded that the sex ratio of orchids growing on unshaded dead trees was significantly female biased. For the duration of the study, most individuals (70%) remained the same sex. In contrast, the sex ratio of orchids growing on shaded live trees was 1:1. A shading experiment of developing inflorescences showed that light, not substrate type was the proximal cue determining sex.

A study of the dioecious *Spinacia oleracea* concluded that drought stress caused sex ratios to be male-biased (Freeman and Vitale 1985). *S. oleracea* seedlings were sown in a common environment, but with different moisture treatments. In dry treatments there were significantly more males than females, and females from the dry treatments were more water stressed than females from wet treatments. Relative levels of the

feminizing hormone, cytokinin, may cause *S. oleracea* to favor maleness over femaleness. Itali and Vaadia (1970) found that under water stress, cytokinin transport out of the root, where it is synthesized, is halted or impeded (Itai and Vaadia 1970). Thus, Freeman and Vitale (1985) argue that it is possible for a *S. oleracea* individual to be male under some environmental conditions and female under other conditions (Freeman and Vitale 1985).

ESD may also occur in gynodioecious taxa if the sex is environmentally plastic (Delph 2003). As stated previously, hermaphrodites of some species may reduce female function under poor conditions to become cryptic males (Lloyd 1976, Delph 2003). Theoretically, hermaphrodites that set less seed have a higher fitness relative to other hermaphrodites when conditions are stressful due to both decreased mortality and increased pollen production (Lloyd 1976, Charlesworth and Charlesworth 1978). In this case ESD may often be confounded with selection (environment-biased mortality and selection) because both mechanisms may produce hermaphrodites that reduce seed set under poor conditions. The distinction is that in ESD outside stimuli causes a plant to be a particular sex. Many studies presented here do not make distinctions between ESD and selection. A common garden experiment involving clones is necessary to untangle ESD from selection. Thus, the following examples do not clearly distinguish between ESD and environment-biased selection for hermaphrodite morphs. Hermaphrodite fruit set has been studied in *Hebe strictissima*, *Ochradenus baccatus*, and *Silene acaulis* (Delph 1990, Wolfe and Shmida 1997, Delph 2001).

Hermaphrodites of *Hebe strictissima* showed a significantly positive relationship between fruit set per shoot and plant vigor (measured by leaf: shoot ratio), which was correlated with site quality, indicating that hermaphrodites might be able to reduce fruit set under poor conditions (Delph 1990, Delph 2003). This is evidence for ESD because fruit set in hermaphrodites is dependent upon plant vigor, which is linked to environment. However, it is also possible that there has been selection for hermaphrodite cohorts to set less fruit in poor quality sites.

The semideciduous subdioecious shrub, *Ochradenus baccatus*, grows in Israel and has populations composed of females, hermaphrodites, and male morphs that have non-functioning pistils (Wolfe and Shmida 1997). A survey of 24 populations along a 350 km north-south transect found that hermaphrodites were more common than male morphs in populations with greater rainfall. The authors found evidence that the ability for hermaphrodites to set seed and produce pollen was partly attributed to plant size (vigor). This result suggests ESD may be important, as in *H. strictissima* (Delph 1990).

A population study of sex-ratio variation in *S. acaulis* found that female frequency was significantly higher in sites in which hermaphrodites had low seed fitness (Delph and Carroll 2001). The lack of congruence between site quality and reduced female fertility in hermaphrodites of *S. acaulis* suggests that selection, and not ESD, may be important. Thus, reduced seed set in hermaphrodites may be a trait that is selected for over many generations and not a plastic environmental response.

Population dynamics of parental sex-determining genes

The genetics that govern sex inheritance may shape SSS. Populations of gynodioecious species with CMS may produce exhibit female-biased sex ratios when nuclear genes that can restore male fertility are absent from local neighborhoods (Frank 1989). In taxa with CMS genes, the most conspicuous cases of female spatial segregation have been found in recently founded populations.

Disturbance may open up new habitats for colonization. For instance, fire was found to generate founder effects in populations of *Thymus vulgaris* (Manicacci et al. 1996). Young populations of *T. vulgaris* had very high frequencies of females (~90%) spatially segregated into clusters. Female clusters often contained a single mitochondrial allele (mitotype), an indication that all females were descended from a single mother, had local seed dispersal, and were fertilized by pollen from a limited hermaphrodite source population lacking the appropriate nuclear male fertility restorers. Furthermore, neighboring populations only several kilometers apart usually contained female clusters with different mitotypes, indicating that seed dispersal was on the order of a few meters (Manicacci et al. 1996).

Disturbance also affected the population genetics of *Beta vulgaris maritima* (Laporte et al. 2001). Founder effects were evident (low cytoplasmic diversity and high female frequencies) among French populations of *Beta vulgaris maritima* living near the sea (Laporte et al. 2001). Populations of *B. vulgaris maritima* near the sea were occasionally lost due to wave action, and new populations would replace the old populations. When these populations were young, cytoplasmic diversity was low and

female frequency was high (Laporte et al. 2001).

Populations of *Silene latifolia* exhibited highly skewed sex ratios. *S. latifolia* has a X/Y sex determination system, but sex-ratios among some related individuals can be highly female-biased (80-100 % female) and produce highly-female biased populations (Taylor 1994, Taylor 1999). Female-biased sex ratios in some families of *S. latifolia* appear to be caused by particular associations between alleles on the X- and Y-chromosomes (Taylor 1994). Taylor and Ingvarsson (2003) suggest that deleteriousY-linked alleles are masked by some X-linked alleles. Males that carry X-linked alleles that do not mask deleterious alleles may experience a transmission disadvantage resulting in an overall reduction of male gametes or male progeny.

It is also possible that female-biased ratios in *S. latifolia* are due to abortion of Y-bearing pollen (Taylor and Ingvarsson 2003). This explanation is consistent with the observation that male sires that produce female-biased sex ratios experience higher rates of pollen abortion than male sires that produce non-biased sex ratios (Taylor and Ingvarsson 2003). In crosses with unlimited pollen, males that produce female-biased broods can produce as many offspring as males that produce equal sex ratios (Taylor 1999). However, in pollen competition experiments pollen that produces female-biased broods produces fewer offspring of both sexes (Taylor 1999). Thus, when pollen is limiting, males of *S. latifolia* may experience a transmission disadvantage compared to males that produce non-biased sex ratios (Taylor and Ingvarsson 2003).

In conclusion, many mechanisms can cause SSS and sex-biased ratios. Other factors besides, or in addition to CMS, may generate female-biased sex ratios seen in

Silene acaulis. However, the highly female-biased sex ratios seen in *S. acaulis* strongly suggest CMS involvement. This study did not explore all the mechanisms that can lead to SSS, but merely sought to find spatial patterns within populations that are consistent with other species that have CMS. Therefore, ecological and environmental differences among populations may play a role in generating differences in sex ratios, as was found in *Plantago lanceolata* (Krohne et al. 1980). Also, if *S. acaulis* has ESD, it may not play a large role because few plants were found to change sex due to environment in this study (maximum 10 %) (Table 1).

Specific Introduction

Non-random spatial distributions of females and hermaphrodites are commonly observed within gynodioecious plant species (Belhassen et al. 1991, Manicacci et al. 1996, Laporte et al. 2001, Olson and McCauley 2002, Keller 2002). The genetics of sex determination has been implicated as a causal factor generating spatial segregation of the sexes and theoretical studies suggest that spatial segregation of the sexes, in turn, may help maintain a gynodioecious sexual system (Frank 1989, Bellhassen et al. 1993, McCauley 1994, Manicacci et al. 1996, McCauley and Taylor 1997, Tarayre et al. 1997, McCauley and Brock 1998, McCauley et al. 2000, Laporte et al. 2001, McCauley et al. 2001, and Olson and McCauley 2002). In this study, we report on the spatial patterns of gender and maternally inherited genetic markers within and between populations in the long-lived gynodioecious plant, *Silene acaulis*. These patterns provide insight into the presence and prevalence of non-random spatial associations between genders, the scale of seed dispersal, and the potential for ecological factors associated with spatial segregation of sexes to influence the maintenance of gynodioecy.

In many well-studied gyndioecious species, sex is determined by a combination of genes inherited from the mitochondria and nuclear genome (Hanson 1991). Maternally inherited mitochondrial genes have been linked to the production of non-functional pollen and are called cytoplasmic male sterility (CMS) genes (Schnable and Wise 1998).

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submitted to *International Journal of Plant Sciences*

Nuclear male fertility restorer genes reinstate male function and the hermaphrodite phenotype. Crossing studies indicate that nuclear male fertility restorers at a particular locus restore only one CMS type; this pattern is consistent with the CMS and restorer genes interacting in a gene-for-gene fashion (Charlesworth and Laporte 1998, Schnable and Wise 1998). Hereafter, we will refer to nuclear male fertility restorers as "appropriate" when nuclear restorers are matched with the correct CMS genes for expression of the hermaphroditic phenotype.

Theoretical studies suggest that females may spread when the appropriate restorers for the CMS genes that they carry are not present within the local population (Frank 1989, Gouyon et all. 1991). This process is comprised of two elements. First, females in gynodioecious species usually enjoy an advantage in seed production compared to hermaphrodites, when females reallocate resources not used in pollen production to produce more seeds (Gouyon et al. 1991, Ashman 1994, Delph 2001), and when hermaphrodites produce fewer or inferior seeds compared to females due to inbreeding depression (Hanson 1991). Second, when local pollen sources contain low frequencies of the appropriate nuclear male fertility restorers for the CMS types carried by females, proliferation of females may occur because progeny from female mothers are largely female. Across populations this "epidemic" spread may generate high variation in sex ratios (Delph 1990, Hermanutz and Innes 1994, Tarayre et al. 1997, Morris and Doak 1998, Laporte et al. 2001, Keller 2002, and Olson and McCauley 2002, this study) and within populations it may generate small patches of female individuals (Belhassen et al. 1991, Manicacci et al. 1996, Laporte et al. 2001, Olson and McCauley 2002, Keller

2002).

The spread of females is restricted by pollen limitation and by the arrival of nuclear male fertility restorers within populations by gene flow or mutation. Pollen limitation may reduce the fitness of females when female frequencies become sufficiently high that females produce fewer seeds because of the lack of nearby hermaphrodite pollen sources (Graff 1999, McCauley et al. 2000). The action of pollen limitation is supported by studies in *Sidalcea malviflora malviflora* and *S. vulgaris*, wherein females had lower seed production when hermaphrodites were rare (Graff 1999, McCauley et al. 2000). Additionally, the proliferation of females can be restricted when the appropriate nuclear restorers arrive in a population (Gouyon et al. 1991, Frank 1989). Because females must receive pollen from hermaphrodites, female advantage from increased seed production may be mitigated in the next generation if some of their offspring are restored to the hermaphrodite state. Some studies of gynodioecious species have found decreasing female frequencies over time, which is predicted when nuclear restorers spread through populations (Krohne et al. 1980, Dommee and Jacquard 1985, Tarayre et al. 1997, Keller 2002).

Local seed dispersal may generate a non-random spatial distribution of maternal lineages within a population; in gynodioecious species this could result in the observation of clusters of individuals with genetically distinct cytoplasmic types and perhaps, clusters of females. Clusters of individuals with homogeneous cytoplasmic types have been reported in some gynodioecious species. *Thymus vulgaris* had very low diversity of cytoplasmic genes associated with females within clusters ranging in size from a few

meters to 20 meters and high diversity of cytoplasmic genes among clusters (Manicacci et al. 1996). Studies have also found reduced cytoplasmic diversity in female clusters of *Thymus vulgaris* (Belhassen et al. 1991), *Beta vulgaris maritima* (Laporte et al. 2001), and *Silene vulgaris* (Olson and McCauley 2002). However, cytoplasmic clustering does not necessarily extend to sex expression because nuclear male fertility restorers are biparentally inherited and may be randomly distributed across a population, even if cytoplasmic types are not. Thus, when the appropriate restorers are present within populations, some fraction of the offspring from a female will be hermaphrodites. In this case, cytoplamic types, but not sex, may be clustered. This situation was found in Virginian populations of *S. vulgaris* (Olson and McCauley 2002).

Silene acaulis is a gynodioecious species common in arctic and alpine ecosystems. It is long-lived, sometimes reaching ages in excess of 300 years (Morris and Doak 1998) and thus may be particularly amenable to displaying changes in sex ratio across age-classes (through time). Females enjoy greater seed set compared to hermaphrodites (Delph and Carroll 2001) and the presence of several mitochondrial types allows the identification of different maternal lineages that may co-segregate with CMS types (Städler and Delph 2002, this paper). Keller (2002) found fine-scale spatial clustering of *S. acaulis* females in Alaskan populations as well as a directional trend for females to be in larger size classes (older individuals), an observation that is consistent with the spread of nuclear male restorers within populations.

Seed dispersal in *S. acaulis* is unspecialized and seeds probably fall close to their mothers. This local seed dispersal is likely to generate patterns where neighboring plants

are more likely to be maternal siblings than individuals drawn at random from the population. Small-scale genetic structure for nuclear genes in Colorado populations of *S. acaulis* supports these predicted patterns (Gehring and Delph 1999), but maternally inherited markers may be a better indicator of the presence and scale of sibling patches. By extension in species with CMS, clusters of females may result when maternal plants do not receive pollen with appropriate nuclear male fertility restorers for the CMS genes they carry. Keller (2002) observed female clusters in three of six *S. acaulis* Alaskan populations.

It is not currently known whether gynodioecy in *S. acaulis* results from CMS, but CMS is present in the congener species, *S. vulgaris* (Olson and McCauley 2002) and gynodioecy is thought to be a characteristic of the basal lineages of the genus *Silene* (Desfeux 1996). Additionally, some populations of *S. acaulis* exhibit highly female-biased sex ratios, indicative of maternal control of male sterility (Hermanutz and Innes 1994, Morris and Doak 1998, Keller 2002, this study).

This is the first study to investigate the spatial and temporal structure of mitochondrial and chloroplast alleles, their relationship with each other, and their associations with sex expression in *S. acaulis*. We use mitochondrial (mt.) and chloroplast (cp.) alleles to assess cytoplasmic diversity among individuals from five Alaskan populations of *S. acaulis* distributed across two mountain ranges. A small number of plants from Kennicott, Alaska in the Wrangell Mountains and Finse, Norway were included in this study to assess the cytoplasmic variation of *S. acaulis* across large geographic regions.

In this study we explore the associations for sex and cytoplasmic genes and the spatial and temporal patterns of sex and cytoplasmic types within populations. We have six main questions, several of which are predictions that arise from joint CMS-nuclear sex determination. First, what is the level of chloroplast and mitochondrial polymorphism within and among populations? Second, how does female frequency vary among populations and how is this variation consistent with cytoplasmic-nuclear sex determination? Third, is there a 1:1 relationship between sex and cytoplasmic markers as expected when appropriate nuclear male fertility restorers are absent from populations? Fourth, are females and mitotypes patchily distributed, as expected when male fertility restorers are locally absent and females with unrestored CMS types spread seed locally? Fifth, do mitotype frequencies and sex ratios change over time, as expected when mitotypes or nuclear male fertility restorers spread within populations? Lastly, do mitotype and chlorotype associations indicate that both genomes have strict maternal inheritance?

Materials and methods

Study organism

Silene acaulis (L.) Jacq. (Caryophyllaceae) is a gynodioecious, early successional arctic-alpine species with a circumpolar distribution. It is often found on scree slopes and rock crevices, along streams and in tundra on well-drained soils with low organic content (Griggs 1956, Aiken et al.1999). *S. acaulis* is a long-lived perennial (> 300 yrs, Morris and Doak 1998) that forms dense cushions of rosettes and bears pink flowers from early June to late July in Alaska. The flowers have a nectary at the base of the petals and are pollinated by *Bombus* spp. and dipterans (Swales 1979, Alatao 1997, Morris and Doak 1998, personal observation). Hermaphrodites can self-fertilize but are protandrous, producing two whorls of anthers that dehisce prior to style elongation. Females have varying degrees of repressed stamens and do not produce viable pollen. Seeds are housed in capsules that project over the cushion and do not have a specialized mechanism for dispersal.

The genus *Silene* contains several hundred species with diverse breeding systems, including dioecy (males and females), gynodioecy (hermaphrodites and females), hermaphroditism, and trioecy (sexes occur along a continuum to include hermaphrodites, females and males) (Shykoff 1988, Alatalo and Molau 1995, Atalo 1996, Desfeux et al. 1996, and Maurice et al. 1998). Phylogenetic reconstructions of the genus show evidence for an ancestral gynodioecious state with hermaphroditism and dioecy both arising at least twice (Desfeux et al. 1996). Two subspecies of *S. acaulis* are recognized in Alaska: subspecies *exscapa* (*acaulis*) (Allioni) DC., which is distributed throughout Alaska and

arctic Canada, Greenland, Iceland, and Eurasia, and subspecies *subaculescens* (F.N. Williams), C.L. Hitchc. and Maquire, which is mostly limited to the Rocky Mountains in the contiguous USA, Canada, and Alaska (Hulten 1968; Shykoff 1988, 1992).

Subspecies *exscapa* is closer to exhibiting dioecy or triocey compared to subspecies *subaculescens*, which has been primarily categorized as gynodioecious. This is because subspecies *exscapa* produces staminate plants with lower proportions of exerted styles and lower fruit sets when compared to subspecies *subaculescens* (Shykoff 1988, Hermanutz and Innes 1994, Maurice et al. 1998, Delph and Carroll 2001). Morphological characters used to differentiate between subspecies *exscapa* and *subacaulescens* include: leaf shape, calyx length and shape, and length of flower stalks (Hulten 1968). Both subspecies appeared to be within all study populations but hybridizations between the subspecies made discrimination impractical (Hulten 1968).

Study populations

Three of the five populations in this study were located in the White Mountains north and east of Fairbanks, Alaska, USA: Eagle Summit (ES: N 65° 29' W 145° 24'), Twelve Mile Summit (TMS: N 65° 23' W 145° 59'), and Steese Highway 108.5 (SH: N 65° 29' W 145° 23') (Fig. 1). In the White Mountains, populations TMS and SH were found on roadcuts along the Steese Highway and ES was 100- 200 m off the Steese Highway on slopes covered with natural alpine tundra vegetation. TMS was separated approximately 30 km from populations ES and SH, which were within 3 km of each other.

Two additional populations, separated by less than 1 km, were located near Bison Gulch in the Alaska Range, outside Healy, Alaska, USA: Bison Gulch I (BGI: N 63° 48' W 148° 58' 33.9") and Bison Gulch II (BGII: N 63° 48' W 148° 58' 53.2") (Fig.1). Population BGI was located on a dry scree slope, and population BGII was located in a boulder field. Populations in the White Mountains are geographically separated from populations in the Alaska Range by approximately 270 km. Populations BGI and ES from this study overlapped Keller's (2002) study sites of BGI and ES, but BGII, TMS, and SH from this study did not overlap Keller's sites. Seeds from two additional regions, Finse, Norway (N 60° 36', E 7° 30') (collected by Amy Carroll) and Kennicott, Alaska in the Wrangell Mountains (N 61° 30', W 142° 50') (collected by Bill Morris and Dan Doak) were also included to determine if individuals from different parts of Alaska and from Norway had genetic similarities.

Mapping spatial structure

Five sub-arctic Alaskan populations of *S. acaulis* were sampled during the 2002 and 2003 flowering seasons (Fig. 1). Sampling plots were designed to include a minimum of 50 individuals per population. Study plots were rectangular and varied greatly in size (100 m^2- 400 m^2) due to differences in population density and total population size (Table 1). The spatial position of each plant with a maximum diameter greater than 4 cm was mapped by determining its distance from two fixed points using sonar-distance measuring devices (Sonin, Inc., Hopewell Junction, NY, USA).

Individuals smaller than 4 cm were not mapped because of sexual immaturity (personal observation).

Sex determination

Each mapped individual was categorized as nonflowering (NF), hermaphrodite (H), female (F), or environmentally labile (L). Females were defined as individuals that only produced flowers with stunted stamens that were shorter than the calyx; hermaphrodites were defined as individuals with only perfect flowers in which at least one stamen protruded past the petals. A few individuals in the field showed evidence of sex switching between the two flowering seasons and were categorized as environmentally labile. At the time of mapping, several rosettes from each plant were collected and stored at 4° C for subsequent molecular analysis and vegetative propagation in the greenhouse.

Vegetative propagation in the greenhouse was performed to sex non-flowering field plants. Failure to determine the sex of non-flowering plants can bias estimates of sex ratios and incorrectly assumes that the spatial patterns of flowering individuals reflect the spatial patterns of the sexes (Eppley 1988). Within two weeks after collection, stems and roots of all plants within plots (even those that were sexed in the field) were submersed in a formula of rooting auxins (Dip 'N Grow, Inc., Clackamas, OR, USA) following the manufacturer's protocol for woody shrubs, and planted in a 1:1:1 perlite vermiculite, and peat mixture in styrofoam cups pierced at the bottom to allow for adequate drainage. Individual cups were arranged into 6 x 6 (cups), (43 cm^2) trays and

placed in humidity tents in the greenhouse for approximately two months. During this time cuttings were misted to prevent drying. Following removal from humidity tents, trays were fertilized and watered as needed and received approximately 24 hours of light a day. Plants were sexed from September until March 2002.

In addition, seeds from individuals from several populations in Kennicott, Alaska from the Wrangell Mountains and from one population in Finse, Norway were planted and sexed in the greenhouse. The spatial locations of these seeds within populations were not determined. These seeds were given the same water and fertilizer treatment as the cuttings and were placed in humidity tents until they sprouted. Leaf and bud tissue from Wrangell Mountains and Norwegian plants were collected and stored at 4° C for subsequent DNA extraction and molecular analysis.

Plants initiated flowering after several months (Sept. 2002). For six months (Sept- March 2002) plants were sexed as female (F), hermaphrodite (H), environmentally labile (L), or gynomonecious (G) (Table 1). Plants sexed in the greenhouse were categorized using the same criteria as plants sexed in the field. A minority of individuals appeared to change sex (L, labile) or have both sexes on one plant (G, gynomonecy) in the greenhouse. Labile individuals expressed different sexes in the greenhouse and the field or between field seasons. Gynomonecious individuals produced varying ratios of both perfect and pistillate flowers. Because these plants had intermediate sexual states, they could not be categorized as either hermaphrodites or females. Using two mitochondrial types for which there were sufficient sample sizes ($n > 5$), we assessed the associations between mitochondrial types and gynomonecious and labile individuals. We

found no associations for mitochondrial types and gynomonecious and labile individuals (Fisher's exact test: $P > 0.05$) (PROC FREQ FISHER, SAS Institute v. 8, Cary, NC, USA).

Chloroplast and mitochondrial variation in Silene acaulis

Cytoplasmic alleles were used to examine fine-scale population genetic structure and larger geographic associations. Rosettes collected in the field were transferred from 4° C to a -80° C freezer in the laboratory. DNA was isolated from samples frozen with liquid nitrogen using a mechanized tissue grinder and Qiagen DNAeasy 96-well kit for plants (Qiagen, Inc., Valencia, CA, USA).

Length polymorphism in an intergenic spacer was chosen for analysis because evolutionary rates of insertions/deletions (indels) is as great or greater than substitution rates in chloroplast introns of *Silene*, and because high rates of variation for indels have been demonstrated within and among plant populations of the congener *S. vulgaris* (McCauley 1994, Ingvarsson et al. 2003). Approximately 2 ng of genomic DNA was used to PCR amplify the chloroplast intergenic spacer between genes *trnH* and *Psba* using universal primers, 5'ACT GCC TTG ATC CAC TTG GC 3' and 5'CGA AGC TCC ATC TACAAATGG 3' (Hamilton 1999). PCR was performed with an annealing temperature of 56.9° C for 30 cycles. Length polymorphism for large (> 10 bp) indels was evaluated by performing electrophoresis on the complete PCR product (approx. 250-290 bp) on a 2- 2.5 % Metaphor (Cambrex, East Rutherford, NJ, USA) gel for 5- 7 hours at 60- 70 volts. To assess sequence differences among different sized PCR products, a

subset of the three *trnH- Psba* chloroplast types (chlorotypes) was sequenced on the ABI 3100 using ABI Big Dye Terminators and standard protocols (Amersham Biosciences, Inc., Piscataway, NJ, USA). Sequences were aligned using Sequencher (Gene Codes, Inc., Ann Arbor, Michigan, USA) default settings. Primer regions were removed from aligned sequences of all chlorotypes.

HindIII RFLP variation in the flanking region of the mitochondrial subunit I of cytochrome oxidase (*COXI*) was assayed by southern hybridization using non-radioactive digoxigenin-d-UTP (DIG)-labeling (Roche, Inc., Indianapolis, IN, USA). Variation is expected to occur in the flanking region of *COXI* gene, and not within the coding region because the coding regions are generally more conserved (Kelchner 2000). The flanking region of the *COXI* gene was chosen as a marker because it detected polymorphism in population studies of *Thymus vulgaris* and *Silene vulgaris* (Bellhassen et al. 1993, Olson and McCauley 2002).

Total genomic DNA was digested overnight with *HindIII*. Following digestion, DNA fragments were separated by electrophoresis on a 0.8 % agarose gel overnight at 25V. Denatured single-stranded DNA fragments were capillary blotted to a positively charged nylon membrane (Roche, Inc.) overnight and following transfer, UV-crosslinked for 1.5 minutes. The membrane was pre-hybridized with dissolved DIG Easy Hyb Granules (Roche, Inc.) at 42° C for 2- 5 hrs. A 1.5 kb *COXI* DIG-labeled probe was constructed by PCR: a 50 µl reaction included 2 µl of *Silene vulgaris* genomic DNA, 50 units of ExpandHigh Fidelity Enzyme mix (Roche, Inc.), 0.2 µmol of each universal *COXI* primer 1.6k R- 5'AAG GCT GGA GGG CTT TGT AC 3' (Cho et al. 1998), F82-

5' CAA AAG TAT GAA AAG CTG GAG G 3' (Bowe et al. 2000), 1x PCR Buffer with $MgCl_2$, 0.1 mmol dNTP, and 0.5x PCR DIG mix (Roche, Inc). The labeled probe was denatured and diluted approximately 100x prior to overnight hybridization at 42° C.

After overnight incubation, the membrane was washed twice with 2x SSC + 0.1 % SDS for 10 min. at 42° C, followed by two more stringent washes with 0.2x SSC + 0.1 % SDS for 20 min at 65° C. CDP-* (Amersham Biosciences, Inc.), a chemiluminescent molecule, that hybridizes to an alkaline phosphatase labeled anti-DIG antibody, allowed for detection of variable restriction sites flanking *COXI* when the hybridized blot was exposed to X-ray film. Mitochondrial types (mitotypes) were scored using the single most intense band. Substoichoiometric bands possibly due to heteroplasmy were found in some digests but were not easily distinguished across all blots and were not used for mitotype determination.

We evaluated the natural genetic variation of individuals at three different scales: within populations, within mountain ranges, and between mountain ranges. Within-population genetic diversity values (H_e) for the mitotypes were calculated according to Nei (1973) using the program Genetic Data Analysis (GDA); $H_e = 1 - \sum_{i=1}^{m} \chi_i^2$, where χ_i is the frequency of mitotype i and m is the number of mitotypes in the population (Lewis and Zaykin 2001). Nei's genetic diversity values (H_e) both across populations and within mountain ranges were calculated using GDA and compared to assess interpopulation variation in allele frequencies (Lewis and Zaykin 2001). Sample size of populations was

unrelated to the diversity of *COXI* mitotypes (PROC CORR PEARSON: SAS v. 8) (Pearson's r = -0.6373, $P = 0.25$).

Population genetic structure for the *COXI* locus was assessed using θ, a statistic analogous to Wright's F-statistics, but for haploid data (Weir 1996). We used θ in this study to assess the degree of genetic variation within populations, within mountain ranges and between mountain ranges relative to total genetic variation. Values for 0 range from 0 to1; 0 implies populations are panmictic and 1 indicates total geographic isolation of populations. The computer program GDA was used to calculate θ (Lewis and Zaykin 2001).

A Mantel test was performed using the program Mantel-Struct (Miller 1999) to assess the independence of pairwise θ estimates (calculated using GDA, Lewis and Zaykin 2001) and geographic distances of populations between mountain ranges. The Mantel test computes the regression coefficient of pairwise θ estimates to geographic distances and calculates the significance of this coefficient with a *p*-value calculated from 10,000 permutations of the semi-matrix of geographic and genetic distances. Mantel r-statistics range from 0→1 and larger r-statistics are generated when there are positive correlations of geographic distance and genetic distance. Significance of the Mantel r-statistic refers to the proportion of the 10,000 permeations leading to a pairwise θ larger or equal to the observed one. Hierarchical Fisher's exact tests were also used to assess the frequency differences in mitotypes both within and between ranges (PROC FREQ FISHER: SAS v. 8).

Ninety-five percent confidence intervals were constructed for mitotype frequencies when a Fisher's test showed differences between mountain ranges or among populations subdivided by mountain range. Confidence intervals were constructed for mitotypes with sufficient sample sizes ($n > 5$) using a log-linear maximum-likelihood ANOVA model (PROC CATMOD: SAS).

Sex ratio variation

All analyses in this section were performed using SAS Institute v. 8, Cary, NC, USA. Sex ratio variation for hermaphrodites and females was assessed between populations and between and within mountain ranges to determine the level of sex ratio variation (PROC FREQ FISHER: SAS v. 8). Previous studies of gynodioecious species have found that sex ratios can vary widely among gynodioecious populations (Delph 1990, and Tarayre et al. 1997, Laporte et al. 2001, Keller 2002, and Olson and McCauley 2002).

Frequencies of females (F), hermaphrodites (H), labile (L), and gynomonecious (B) individuals were calculated for each study population. We performed a Fisher's exact test to determine whether hermaphrodites and females sexed in the greenhouse had similar ratios to those sexed in the field (PROC FREQ FISHER: SAS v. 8). To control for performing multiple tests and reduce the experiment-wide error rate we used a Bonferroni adjustment.

Associations between sex and mitotype

Associations between mitotypes and sex were examined among Alaskan populations of *S. acaulis*. Heirarchical Fisher's exact tests were performed to test for associations between mitotypes and sex, across all populations, among populations grouped by mountain range, and within each population (PROC FREQ FISHER: SAS v. 8). Fisher's exact tests were also used to test associations between mitotypes and sex across populations for the three most common mitotypes: 1, 2, and 6 to determine if populations differed in their associations for mitotypes (PROC FREQ FISHER: SAS v. 8). Confidence intervals were calculated for frequencies of the most common mitotypes sorted by sex (hermaphrodite or female) pooled from all populations. Ninety-five percent confidence intervals were used to show what was contributing to significant differences found using a Fisher's exact test (PROC CATMOD: SAS v. 8).

Within-population spatial associations of sex and mitotype

Spatial autocorrelation employing join-count statistics was used to determine if populations of *S. acaulis* exhibited clustering of both mitotypes and sexes (Fortin and ver Hoef 2002). A join is a pair of plants of the same category state (sex or mitotype) within a distance interval. Observed joins between hermaphrodites and observed joins between females were assessed at 0.25 m distance intervals for all plants in the population to a distance of 10 m. We chose distance intervals of 0.25 m because this distance detected fine-scale clustering of females within *S. acaulis* populations in a 2002 study by Keller. The observed number of joins within a category state were compared to the expected

number of joins within a category state given no population structure. Expected joins were computed by generating a pseudo dataset 5,000 times with random reassignments of the category state, equal to the frequency in the observed population, to mapped locations of individuals within populations. Correlograms depict standard normal deviate scores at 0.25 m distance intervals [(observed # joins-expected # joins)/(standard deviation of expected # joins)] (Fortin and ver Hoef 2002). Standard normal deviate values greater than two standard normal deviates indicate spatial associations with 95 % confidence. Join-counts of mitotypes were calculated in a similar fashion to detect positive spatial autocorrelation; however, distance intervals were adjusted to every 2 m due to smaller sample sizes within mitotype categories. Within populations, mitotypes that were found no more than five times were not used because of insufficient sample sizes. Join–count statistics for associations among sex and mitotypes were computed using a program written by Jonathan Klaas with Interactive Data Language (Research Systems, Inc., CO, USA) (Appendix).

Within-population temporal associations of sex and mitotype

To assess whether sex ratios and mitotype ratios varied with the age of individuals within populations, we used size classes defined by Morris and Doak (1998). *S. acaulis* cushions have been shown to follow a roughly linear growth pattern (Benedict 1989, Morris and Doak 1998). Morris and Doak (1988) calculated an age range for size classes with size-based population matrices incorporating estimates of the average number of births, deaths, and changes in cushion diameter for each size class between two growing

seasons in Kennicott, Alaska populations. Using this method, plants in the largest size class (> 20 cm) in Kennicott study populations exceeded 300 yr in age, with average growth rates of 0.86-1.63 mm/yr (Morris and Doak 1998). In four of five populations we used Morris and Doak's (1998) size classes: 5- 7.5 cm, 7.5- 10 cm, 10- 12.5 cm, 12.5- 15 cm, 15- 17.5 cm, 17.5- 20 cm, and > 20 cm. However, TMS did not have sufficient sample sizes for seven size classes; thus, size classes were grouped as follows: 5- 10 cm, 10- 15 cm, and 15- > 20 cm.

Cochran-Armitage trend tests were used to assess directional change in both sex and mitotype pairs across size classes (PROC FREQ TREND OPTION: SAS Institute v. 8, Cary, NC, USA). The Cochran-Armitage trend test is based on a linear probability model sensitive to detecting trends in chi-square tests. Only mitotypes with sufficient sample sizes were analyzed with trend tests ($n > 5$). In all populations except TMS, only two mitotypes were at sufficient frequencies for the Cochran-Armitage binomial trend test. Three mitotypes were tested for trend within TMS. The Bonferroni adjustment of the critical values was used to reduce the risk of falsely declaring a difference in tests of multiple comparisons. Chloroplast type was the same within all populations, except for four individuals within TMS, making within-population trend tests of chloroplast type impossible.

Associations between mitotypes and chlorotypes

Most angiosperms have predominately maternal inheritance of both the mitochondria and the chloroplast (Birky 1995). A study of *S. vulgaris* found

phylogenetic congruence between chloroplast and mitochondrial haplotypes, implying both genomes are largely maternally inherited (Olson and McCauley 2000). However, some angiosperm taxa have varying degrees of paternal leakage in the chloroplast (Birky 1995). Additionally, a recent study of wheat found paternal leakage of the mitochondria (Hattori et al. 2002). Genetic studies with *S. vulgaris* have indicated that there may be paternal leakage of the mitochondria and/or the chloroplast, or parallel evolution. For instance, in Virginian populations of *S. vulgaris* multiple mitotypes were associated with the same chlorotype (Olson and McCauley 2000), but in European population of *S. vulgaris* multiple chlorotypes also were associated with the same mitotype (Storchova and Olson, in press). The latter observation suggests reversal mutations under strict maternal inheritance for the chloroplast and mitochondria. In this study we analyzed the associations between chlorotypes and mitotypes to determine the relatedness between the two genomes (PROC FREQ FISHER, SAS v. 8).

Chloroplast types differed for mitotypes 1 and 6 among the White Mountain and Alaska Range populations. To assess whether mitochorndial genomes were homogeneous within both mitotypes 1 and 6, a mitochondrial gene, *Apocytochrome b*, was sequenced in a small subset of individuals (7) across Alaskan and White Mountain ranges. *Apocytochrome b* sequences were compared to sequences from Städler and Delph's (2002) study. *Apocytochrome b* was amplified with approximately 2 ng of DNA using primers at the ends of the gene, 5' GAT TAT CCA ACC CCG AGC 3' and 5' GAA TGG GCG TTA TGG C 3'. PCR was performed with an annealing temperature of 50° C. Samples were sequenced on the ABI 3100 using ABI Big Dye Terminators (Amersham,

Inc.) and standard protocols. Sequences were aligned with Sequencher (Gene Codes, Inc., MI) using default settings.

Results

Chloroplast and mitochondrial variation in Silene acaulis

Three different chlorotypes were distinguished in the 436 individuals screened in this study (Fig. 2). The chlorotypes differed in size due to two different insertions in the same region of the sequence (Table 2). The presence and frequency of different chlorotypes significantly differed across mountain ranges (Fisher's exact test: $P < 0.0001$), but not across populations within mountain ranges (Fisher's exact test: $P = 1.0$). Chlorotypes A and C were found only within the White Mountain populations, whereas chlorotype B was found only in the Alaska Range (Figure 3). In addition, all four individuals from the Wrangell Mountains, Alaska, and the four individuals from Norway carried chlorotype B.

Seven mitotypes were found in Alaskan populations in this study (Figs. 3, 4). Overall Nei's genetic diversity index (H_e) for mitotypes was 0.69 (Table 3). Both genetic diversity, H_e, and genetic subdivision, θ, were lower in the Alaska Range than in the White Mountains (Table 3). Frequencies and types of mitotypes differed between the White Mountains and Alaska Range (Fig 3; Fisher's exact test: $P < 0.0001$). Populations within the Alaska Range were very similar for mitotypes (Fisher's exact test, $P = 0.46$), whereas White Mountain populations were significantly less similar genetically (Fisher's exact test, $P < 0.0001$). Ninety-five percent confidence intervals for mitotypes within populations in the White Mountains show that the population TMS differs significantly in mitotype frequency from the other White Mountains populations (Fig. 5). Within populations, genetic diversity, H_e was highest in TMS ($H_e = 0.65$) and lowest within ES

(H_e = 0.32), both of which are populations in the White Mountains (Fig. 3, Table 4). Pairwise genetic distances (as measured by θ) between populations ranged from 0 to 1 (Table 5). There was no support for isolation by distance (Mantel r = 0.3385, P = 0.21).

Of four individuals screened from the Wrangell Mountains, Alaska, three carried mitotype 4 and one had mitotype 6. In Norway, two individuals were found to have mitotype 2 and another individual had mitotype 4. Two additional mitotypes were found in Norwegian samples that were not present in Alaskan populations; one exhibited a band at approximately 3645 bp and the other at approximately 5570 bp.

Sex ratio variation

Within all populations except BGII, the frequencies of hermaphrodites and females determined in the field and the greenhouse were in agreement (Fisher's exact tests: TMS: P = 1.00; ES: P = 1.00; SH: P = 0.68; BGI: P = 0.46; BGII: P = 0.02). When sexed in the field BGII plants were more likely to be female than when we grew clones in the greenhouse. We attribute this difference to the low number of individuals sexed in the field compared to the greenhouse (Table 1). Therefore, we grouped individuals sexed in the field and the greenhouse for all analyses.

Among Alaskan populations of S. acaulis sex ratios ranged from 36- 83 % F (Fig. 3, Table 1). Population sex ratios differed between mountain ranges (White Mountains, 36- 51 % F vs. Alaska Range, 73- 83 % F; Fisher's exact test among all populations: $P < 0.0001$), but sex ratios among populations within mountain ranges did not differ significantly (White Mountains, P = 0.19; Alaska Range, P = 0.40).

Associations between sex and mitotype

A significant non-random association between mitotype and sex was detected when individuals in all populations were combined (Fisher's exact test, $P < 0.0001$). Ninety-five percent confidence intervals show that mitotype 1 was significantly associated with females (Fig. 6). The other mitotypes with sample sizes sufficient for the CATMOD analysis were not significantly associated with either sex (CATMOD, $P > 0.05$). When we tested for mitotype and sex relationships within the mountain ranges there were no significant associations of sex and mitotype (Fisher's exact tests, White Mountains: $P = 0.17$; Alaska Range: $P = 0.16$). Additionally, within each of the five populations we found no significant association between sex (H and F) and mitotype (Fisher's exact tests, ES: $P = 0.40$; TMS: $P = 0.51$; SH: $P = 0.51$; BGI: $P = 1.00$, BGII: $P = 0.06$). For the three most common mitotypes the associations of sex ratios and mitotypes did not differ across populations (Fisher's exact test: mitotype 1, $P = 0.47$; mitotype 2, $P = 1.0$; mitotype 6, $P = 0.74$).

Within-population spatial associations of sex and mitotype

No trends for female clustering at small distance classes were found within any population (Figs. 7, 8). Within the ES and TMS populations, the spatial positions of mitotypes were autocorrelated up to 2 m, but mitotypes were not clustered within populations SH, BGI, and BGII (Figs. 9, 10). Within ES, mitotype 6 was clustered at distance classes less than approximately 2 m and repulsed at distance classes greater than 6 m. Mitotype 2 in ES followed the same trend as mitotype 6, but more weakly. In the

TMS population, mitotype 5 exhibited weak clustering at distances less than 2 m and greater than 10 m.

Within-population temporal associations of sex and mitotype

No significant trends between sex ratio and size class were found (Cochran-Armitage trend tests, Bonferroni-adjusted $\alpha = 0.01$: ES: $Z = 0.68$, $P = 0.50$; TMS: $Z = 2.03$, $P = 0.04$; SH: $Z = 0.75$, $P = 0.45$; BGI: $Z = -0.57$, $P = 0.57$; BGII: $Z = 0.84$, $P = 0.40$). Also, no significant trends were detected among pairwise comparisons of mitotype frequencies and size classes (Fig. 11). Only pairwise comparisons with sufficient sample sizes were tested ($n > 5$) using Cochran-Armitage analyses: ES, mitotypes 2 vs. 6: $Z = -0.74$, $P = 0.46$; TMS, mitotypes 1 vs. 4: $Z = -1.93$, $P = 0.05$; TMS, mitotypes 4 vs. 5, $Z = 1.40$, $P = 0.16$; TMS, mitotypes 1 vs. 5: $Z = 0.35$, $P = 0.72$; SH, mitotypes 2 vs. 6: $Z = 0.43$, $P = 0.87$; BG1, mitotypes 1 vs. 6: $Z = 0.57$, $P = 0.57$; BGII, mitotypes 1 vs. 6: $Z = 0.57$, $P = 0.57$; Bonferroni adjusted $\alpha = 0.007$).

Associations between mitotypes and chlorotypes

Mitotypes were significantly non-randomly associated with chlorotypes (Fisher's exact test, $P < 0.0001$; Table 6). Chlorotype B was found associated only with mitotypes 1, 6 and 7 and chlorotype C was found associated with only mitotypes 1 and 4, whereas chlorotype A was associated with all mitotypes except mitotype 7. The significant association between mitotypes and chlorotypes appears to be generated mainly due to differences among mountain ranges for both mitotypes and chlorotypes (Table 6).

Interestingly, all combinations of chlorotypes B and A and mitotypes 1 & 6 were common within the surveyed populations. These patterns are indicative of either paternal inheritance of one cytoplasmic compartment and not the other at some time in the history of these lineages, or independent evolution of the same marker in two different lineages.

Mitotypes 1 and 6 were observed in individuals in both the Alaska Range and the White Mountains (Fig 3). If mitotypes1 and 6 from the Alaska Range arose via independent evolutionary events (homoplasy), we expect that individual with these two mitotypes would differ at other mitochondrial loci. We tested whether individuals with mitotypes 1 & 6 in the Alaska Range and the White Mountains also shared alleles at the *Apocytochrome b* mitochondrial locus studies by Städler and Delph (2002). We sequenced 980 b.p. of Apocytochrome b from 4 individuals from the Alaska Range and 3 individuals from the White Mountains (Table 7). Mitotype 1 from *COXI* hybridizations was associated with a single *Apocytochrome b* haplotype, S-AR-3, even when individuals were screened from different mountain ranges.

However, mitotype 6 was associated with two different *Apocytochrome b* haplotypes suggesting either this RFLP mitotype is very old and the *Apocytochrome b* gene has accumulated variation since it arose or RFLP mitotype 6 arose multiple times. Close re-examination of the *COXI* blot revealed a slight size difference (< 1 mm) between the individual with *Apoctochrome sequence* SHIFT-6 and the two individuals with S-AR-2. Because the size difference was so small we decided that this difference could not be detected with reliability. These results indicate that *COXI* mitotype 6

contains some individuals with different *Apocytochrome b* sequences and marks different lineages.

Discussion

Chloroplast and mitochondrial variation in Silene acaulis

Three chlorotypes were found in a survey of one intergenic spacer region in this study of *S. acaulis*. Genetic studies of *S. vulgaris* from the U.S. have found similar levels of chloroplast polymorphism among populations (McCauley et al. 2000, Olson and McCauley 2000). However, a study comparing chloroplast polymorphism of *S. acaulis* as a function of latitude found very low diversity of chlorotypes (Abott et al. 1995). In Abott's study (1995) only two chlorotypes were resolved after RFLP analysis with nine chloroplast probes. Perhaps RFLP variation in *S. acaulis* is more easily identified in intergenic regions than exons.

We found nine different southern blot RFLP mitotypes among Alaskan and Norwegian populations of *S. acaulis*. Two mitotypes were found only in the Norwegian populations, but Norwegian populations also shared mitotypes 2 and 4 with the Alaskan populations. The level of mitochondrial variation found across populations ($\theta = 0.4247$) was comparable to other studies of gynodioecious species that had θ values ranging from 0.21- 0.54 (Bellhassen et al. 1993, Cugen et al. 1994, DeHann et al. 1997, Groenendijk et al. 1997, Olson and McCauley 2002).

Clear geographic structuring of chlorotypes and mitotypes occurred across mountain ranges. Chlorotypes found in the two mountain ranges were completely different, and only two of the seven mitotypes were present in both ranges (Fig. 3). The presence of mitotypes 1 and 6 in the Alaska Range and White Mountains and the presence of mitotype 6 in the Wrangell Mountains indicate that either past or current gene

flow connected these populations. Or, these mitotypes were present in a common ancestral population and were maintained in all populations during the migration to their present locations. However, mitotype 4 was found in the White Mountains and in the Wrangell Mountains, but not in the Alaska Range. This result, coupled with the fact that the Alaska Range populations have low mitochondrial genetic diversity, may imply that only only a few individuals founded Alaska Range populations. Additionally, the Alaska Range populations are very close to one another (~1 km) and probably do not reflect total mitochondrial genetic diversity for the entire Alaska Range.

Unlike the mitochondrial affinities between individuals in the White and Wrangell Mountains, the same chloroplast DNA types were found in the Alaska Range, the Wrangell Mountains, and Norway, but not found in the White Mountains. This may be an indication of past and present geographic isolation of White Mountain populations. Presently, gene flow between White Mountains and Alaska Range is somewhat restricted by 200 km of Tanana River floodplain. Additionally, the Alaska Range and Wrangell Mountains are more proximal to each other than to the White Mountains and form a more continuous alpine habitat, in comparison to the Tanana River Valley that currently bisects the White Mountains and other mountain ranges. Thus, it is possible that higher rates of gene flow between the Alaska Range and the Wrangell Mountains and isolation of the White Mountains has created present-day chlorotype patterns.

Sex ratio variation and associations between sex and mitotype

Populations in the Alaska Range had higher female frequencies than those in the White Mountains. Population-level studies of gynodioecious species have shown that sex ratios can vary widely even among nearby populations (Delph 1990, and Tarayre et al. 1997, Laporte et al. 2001, Keller 2002, Olson and McCauley 2002). Differences in sex determining genes could be one reason why mountain ranges had different sex ratios. When all populations were pooled, mitotype 1 was significantly associated with females and was more common in the Alaska Range. Across mountain ranges, females did not significantly differ in their associations with mitotype 1. Therefore, if mitotype 1 is associated with the same CMS type in the White Mountains and the Alaska Range, then differences in the frequency of mitotype 1 across mountain ranges may be partly responsible for differences in sex ratios across mountain ranges. Although I did not test for selection, it is also possible that there has been selection for females in the Alaska Range, which has caused mitotype 1 to be more abundant in the Alaska Range than the White Mountains. For instance, females may be selected for if pollen limitation is less severe in the Alaska Range than the White Mountains and, as a result, the ratio of female: hermaphrodite seed production is higher in the Alaska Range than the White Mountains (McCauley et al. 2001). Other environmental differences (i.e., site quality) among mountain ranges may also affect female frequency. Future studies may want to address issues regarding how environment affects sex ratios across Alaska.

Within-population spatial associations of sex and mitotype

Two types of spatial patterns were evident within populations: clustering of mitotypes, but not females; and more often, no clustering of mitotypes or females. Three mitotypes exhibited clustering within patches less than 2m in diameter in two populations of *S. acaulis* in this study (Figs. 8, 9). Because the mitochondrial genome is maternally inherited and because *S. acaulis* does not have a specialized seed dispersal mechanism, this pattern is consistent with the expectation that seed dispersal is limited. This result also suggests that nuclear male fertility restorers influencing sex expression are sufficiently panmictic and break up sex associations in clusters of CMS types. Because *S. acaulis* is insect pollinated, nuclear male fertility restorers, which are transmitted through both pollen and seed, may be more randomly distributed within populations than CMS genes that are spread only through seed. Relative to seed dispersal, pollen dispersal may be more homogeneous within populations and adjacent subpopulations (McCauley 1994, Tarayre et al. 1997, Levy and Neal 1999, Laporte et al. 2001). In support of this hypothesis, chloroplast markers were estimated to have 3x lower rates of gene flow than nuclear markers in both *S. vulgaris* and *S. latifolia* (McCauley 1994, 1998). Also, a population study of *Thymus vulgaris* comparing genetic diversity of cytoplasmic and nuclear markers estimated pollen flow 11-14 times greater than seed flow (Tarayre et al. 1997). Both *S. vulgaris* and *T. vulgaris* are pollinated by flying insects and have unspecialized seed dispersal, similar to *S. acaulis*.

It is also possible that within populations and clusters of *S. acaulis* there is ongoing selection for one sex over the other. McCauley et al. (2001) hypothesized that when females of *S. vulgaris* become frequent, females may become pollen limited, resulting in higher fitness for hermaphrodites that carry appropriate restorers. Because hermaphrodites with appropriate restorers recruit females to have hermaphrodite-biased offspring this may break up clusters of females (McCauley et al. 2001). Because *S. acaulis* is long-lived, and the hypothesized selection does not impact relative survival of the sexes but only reproductive fitness, some remnant of female clusters would be expected to persist as selection breaks up these clusters. In this study we did not find clustering of females or associations of sex and mitotypes within populations. Thus, well-dispersed nuclear restorers may prevent female clusters from forming, and this mechanism may be more important than selection for females due to pollen limitation.

The dominant spatial pattern was no clustering of mitotypes and females. Mitotypes 1 and 4 did not exhibit clustering and three populations had no clustering of mitotypes. We did not find any incidence of female clustering in populations of *S. acaulis*, a result which is surprising, since Keller (2002) found female clustering in three of six Alaskan populations. These results indicate that either the distances at which seeds disperse are sufficiently large that local neighborhoods of maternal half sibs do not develop, or perhaps that progeny from different mothers have higher fitness in the environment close to the mother. Given the exposed environment in which *S. acaulis* is found, it is likely that wind may have played a large role in dispersing their lightweight seeds. Populations in the Alaska Range have a more random distribution of mitotypes

and sex than the White Mountains populations so there may be environmental factors that influence seed dispersal differently among mountain ranges. Additionally, the SH, BGI, and BGII populations were located on steep slopes which seeds may have rolled down.

Within-population temporal associations of sex and mitotype

We found no evidence for an association between mitotypes and sex ratios across size classes consistent with the spread of mitotypes or sex types through time. Our results are in contrast to previous studies of gynodioecious species that have found that female frequency decreases over time, as predicted when nuclear restorers spread through populations (Krohne et al. 1980, Dommee and Jacquard 1985, Tarayre et al. 1997, Keller 2002). Keller (2002) found two White Mountains populations of *S. acaulis* in which the proportion of females significantly decreased over time. Perhaps part of the reason that we found few changes over time was that two of the five populations we studied were along roadsides and appeared to exhibit much higher growth rates than individuals in less disturbed sites. Also, the ability to detect changes in the sex ratio over time may have been dampened in this study because *S. acaulis* is very long-lived with overlapping generations. Plants ~100-300 years old will be breeding alongside younger plants. Old hermaphrodites that lack appropriate restorers and young hermaphrodites that carry appropriate restorers may breed to create non-biased sex ratios.

We recognize that caution is warranted when correlating cushion size with age across populations, especially when populations have different soil types and disturbance histories. In this study, size classes were used as a proxy for cushion age and were

determined using Morris and Doak's (1998) data from the Wrangell Mountains. Our populations probably differed in growth rates compared to the Wrangell Mountains due different soil substrates. Populations TMS and SH in the White Mountains were located in the disturbed soil adjacent to the road (Steese Highway). We estimate that the maximum age of these populations is 77 years, and they may be much younger because the Steese Highway has been widened since 1927. However, some individuals within TMS and SH are very large and are predicted to be >300 years old using the dating parameters of Morris and Doak 1998. Thus, uniform sex ratios and mitotype frequencies over time in roadcut populations may only mean that there has not been enough time for selection to act to change these ratios.

Associations between mitotypes and chlorotypes

Unlike other reports of strong linkage disequilibrium between chloroplast and mitochondria molecular markers (Olson and McCauley 2000, Storchova and Olson unpublished Manuscript), we found a breakdown in linkage in *S. acaulis* (Table 6). In particular, all combinations of mitotypes 1 & 6 and chlorotypes A &B and all combinations of mitotypes 1 & 4 and chlorotypes A & C were present in natural populations. We have some evidence that these patterns result from homoplasy because slight differences in the sizes of bands associated with mitotype 6 were recognized after mitotype 6 was determined to be associated with two different *Apocytochrome b* types. Unfortunately, the slight difference in sizes of bands was not sufficiently large to allow re-scoring of all southern blots. However, it does suggest that southern blot RFLP bands

of the very similar size may be inadvertently scored as the same, thus homoplasy may arise through our inability to detect two marker bands as different. Very similar sized banding patterns may arise independently in the mitochondrial genome as a result of mutation "hotspots" (loci where mutations occur much more frequently than the rest of the genome) in the flanking region of the *COXI* gene. Also, stem-loop structures in the *trnH-Psba* intergenic spacer may also generate reversal mutations leading to homoplasy (Kelchner 2000, Storchova and Olson unpublished Manuscript). Alternatively, paternal inheritance of either the mitochondria or chloroplast could generate the observed breakdown in linkage disequilibrium. Paternal inheritance of the mitochondrial genome has been observed in wheat (Hattori et al. 2002), and paternal inheritance of the chloroplast is not uncommon in angiosperms (Birky 1995).

In summary, we posit that the White Mountain populations may have been isolated from other populations in this study for some time and that the Alaska Range populations may have low genetic variation due to genetic drift due to sampling two proximal populations. The associations between mitotypes and sex indicate that maternal genes do not solely determine sex in Alaskan populations of *S. acaulis*. In contrast, studies of thyme and sea beet have found purely cytoplasmic inheritance of sex (Bellhassen et al. 1993, Cugen et al. 1994, Manicacci et al. 1996, Laporte et al. 2001). In this study mitotypes are rarely clustered, indicating that seeds may be dispersed farther and mixed more randomly in space then previously thought. Also, hermaphrodites and the masked CMS types they carry have not increased in relation to females with increasing population age. From this study it appears that overlapping generations may dampen the

ability to detect sex and mitotype shifts. Lastly, the associations of mitotypes and chlorotypes suggest that there may be paternal leakage and/ or hotspots for mutation in marker genes in this study. Paternal leakage will affect tests used in this study that assume that CMS marker genes have strict maternal inheritance. It is important to ascertain what processes are causing observed patterns to determine if there is leakage of the chloroplast, mitochondria, or both genomes.

CONCLUSIONS

In this genetic study of Alaskan populations of *Silene acaulis* we investigated the variation in the frequencies of cytoplasmic markers and sex across space and time. We found that variability in cytoplasmic markers was differentiated between the two mountain ranges in this study. Chlorotype patterns suggest that the Alaska Range populations are more genetically similar to Norwegian and Wrangell Mountain populations than the White Mountain populations. This result may be due to isolation of the White Mountain populations from other populations in this study. The White Mountains are the most isolated mountain range in the study and they are much older. Because the White Mountains are both geologically older and isolated, we expect that the population's lineages are older and less genetically similar to other populations. Mitotype patterns and the close proximity of populations suggest that the Alaska Range populations do not totally represent the entire Alaska Range and may have low genetic diversity because of founder effects.

Sex ratios were associated with the mountain range in which the population was located. The Alaska Range (80-83 %F) had a higher frequency of females than the White Mountains (36-51% F). We did not find any associations for mitotypes and sex within mountain ranges (Alaska Range and White Mountains) or populations. These results suggest that sex determination in *S. acaulis* is not solely dependent upon maternal factors. Interestingly, mitotype 1 was associated with females when all populations were pooled. Pooling the populations gave the statistical test enough power to detect differences. This

result suggests that mitotype 1 may be contributing to the high female frequency observed in the Alaska Range.

Within populations, mitotypes, but not sex, were clustered at a scale ≤ 2 m. We expect mitotype clustering when seed dispersal is local. The overall spatial pattern of mitotypes suggests that seed dispersal is often not local. This result is surprising because a previous study found female clusters with three of six Alaskan populations (Keller 2002). Female clustering is expected when appropriate nuclear male fertility restorers are locally absent and when seed dispersal is local. In this study, we found three cases in which mitotypes were clustered at two meters but sex was not. Patchiness of mitotypes, but not sex, may be due to differences in dispersal patterns between CMS genes and nuclear male fertility restorers. These results suggest that nuclear restorers are well distributed within populations and prevent female clustering.

Our results suggest that sex ratios and mitotypes have not changed over time. Both sex ratios and mitotype ratios did not change over the time span that we could observe through assessing size classes of plants in our populations. This result conflicts with theories that predict that sex assemblages fluctuate with time (Gouyon et al. 1991, Frank 1989). Empirical studies of *Plantago lanceolata*, *Thymus vulgaris*, and *Silene acaulis* have found that hermaphrodites increase in frequency as the population ages (Krohne et al. 1980, Dommee and Jacquard 1985, Tarayre et al. 1997, Keller 2002). In this study, we conclude that because *S. acaulis* is so long-lived, overlapping generations may dampen the ability to detect any changes in either sex or mitotype ratios.

The associations of mitotypes and chlorotypes in this study differ from previous studies and suggest that there may be paternal leakage of cytoplasmic types or evidence for reversal mutations in the evolutionary past (Olson and McCauley 2000, Storchova and Olson, unpublished Manuscript). The observation that there may have been paternal leakage or reversal mutations could be explored in a future study. A crossing study between parents with polymorphic gene regions could be set up to determine if paternal leakage occurs frequently occurs. Additionally, the likelihood of a reversal could be ascertained by studying the protein and gene structure of the intergenic spacer *trnH-psbA*.

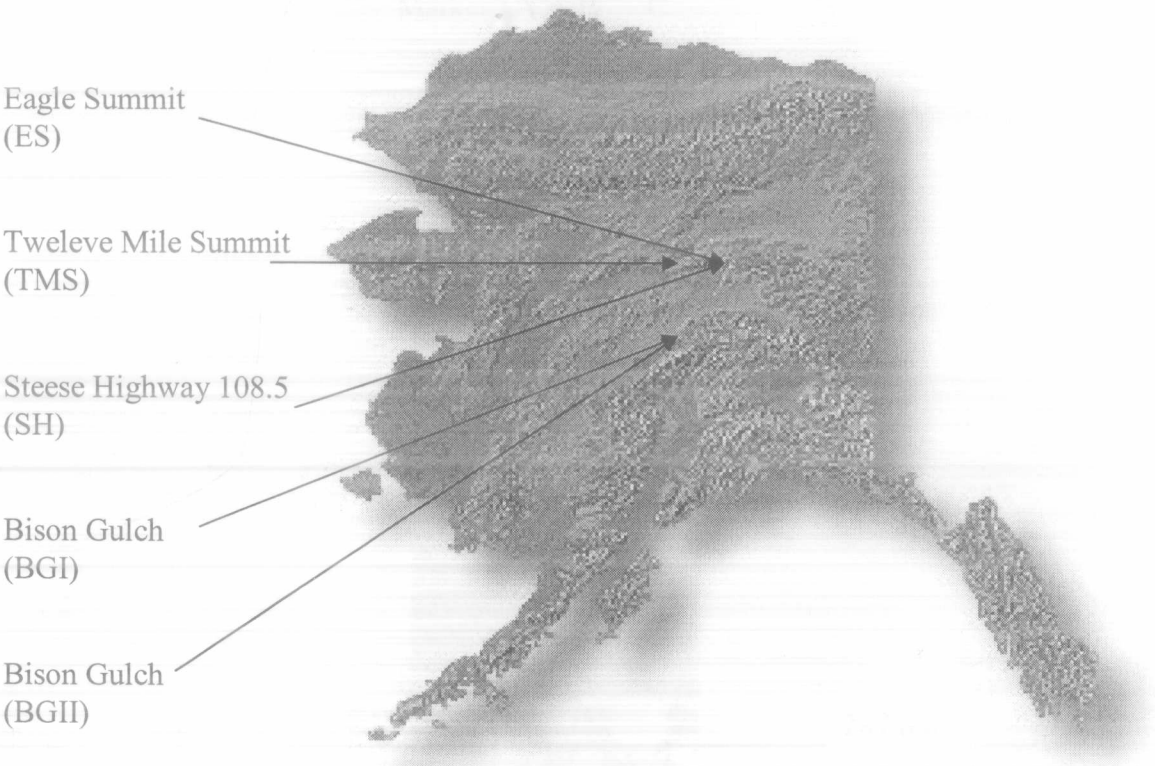

Fig. 1. Map of Alaska, USA, with approximate locations of the five populations in this study.

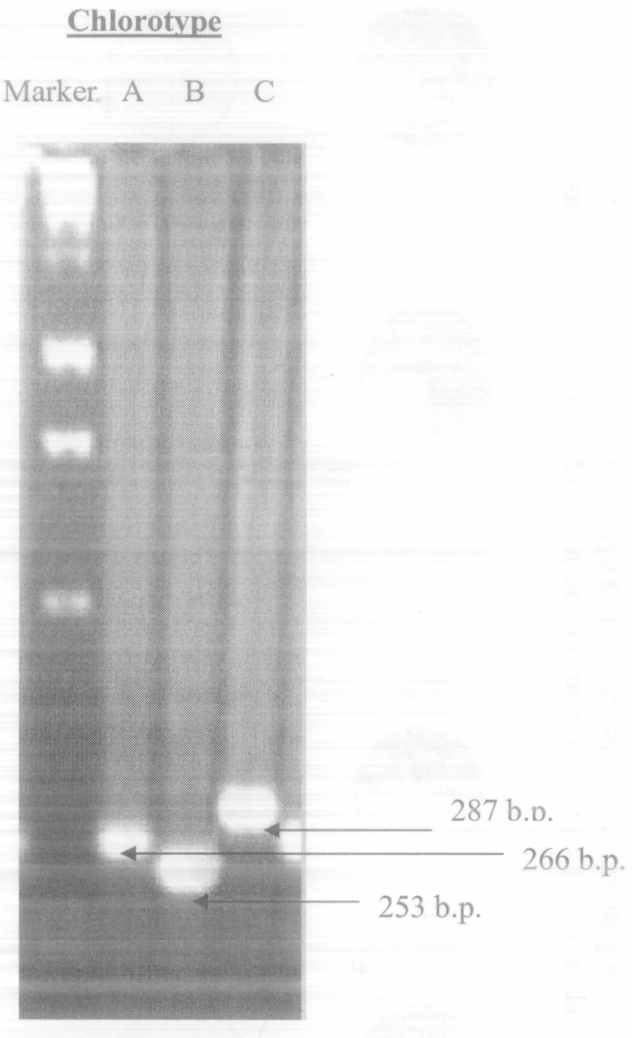

Fig. 2. Size variation of PCR fragments amplified from the chloroplast intergenic spacer, *trnH- Psba*. Three different chlorotypes were distinguished based on size (bp = base pairs).

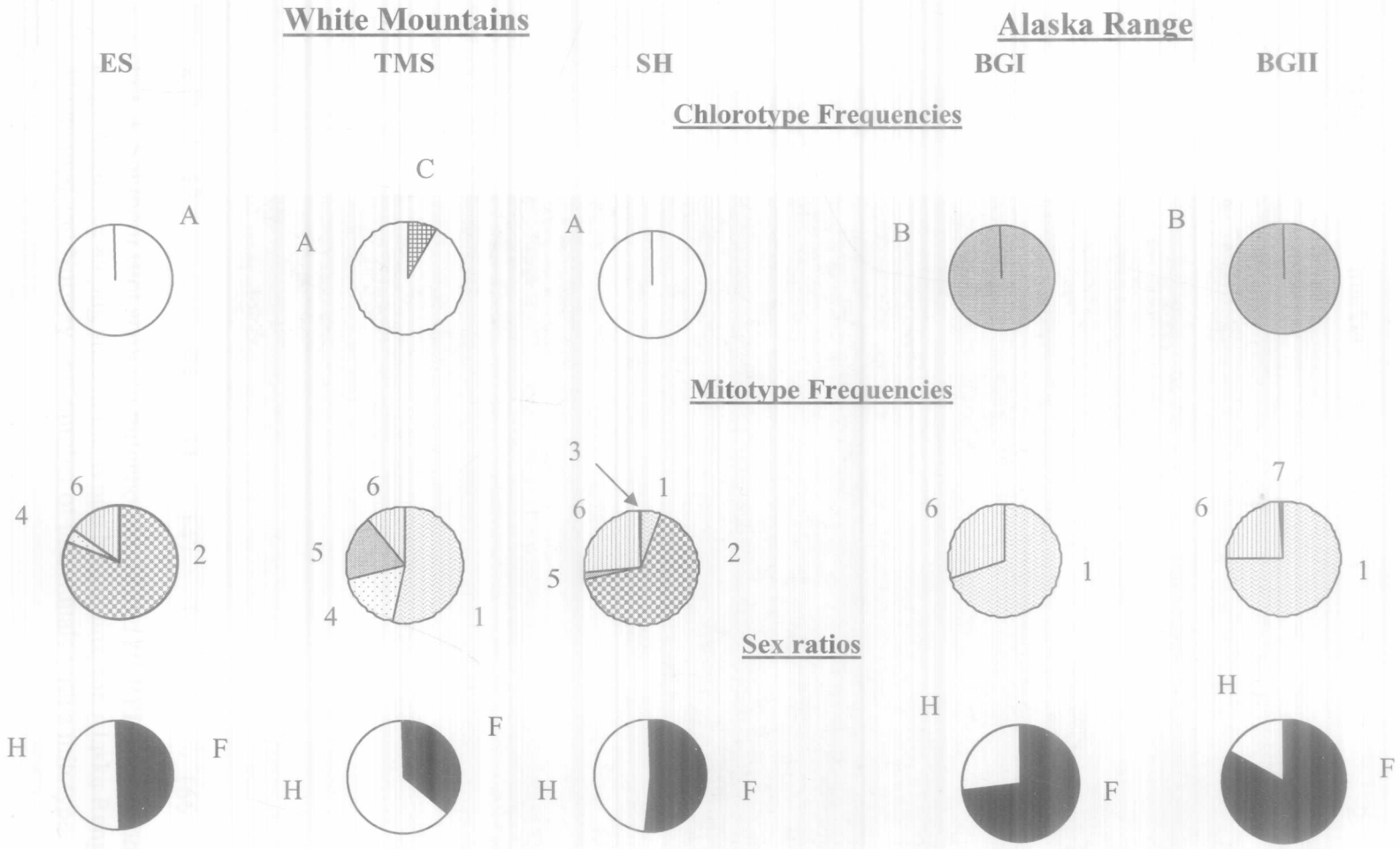

Fig. 3. Distributions of three chlorotypes, seven mitotypes, and sex ratios among Alaskan study populations. ES= Eagle Summit, TMS= Twelve Mile Summit, SH= Steese Highway 108.5, BGI= Bison Gulch I, BGII= Bison Gulch II. Letters A, B, and C designate chlorotypes, numbers 1-7 indicate mitotypes, and H= hermaprodites and F= Females.

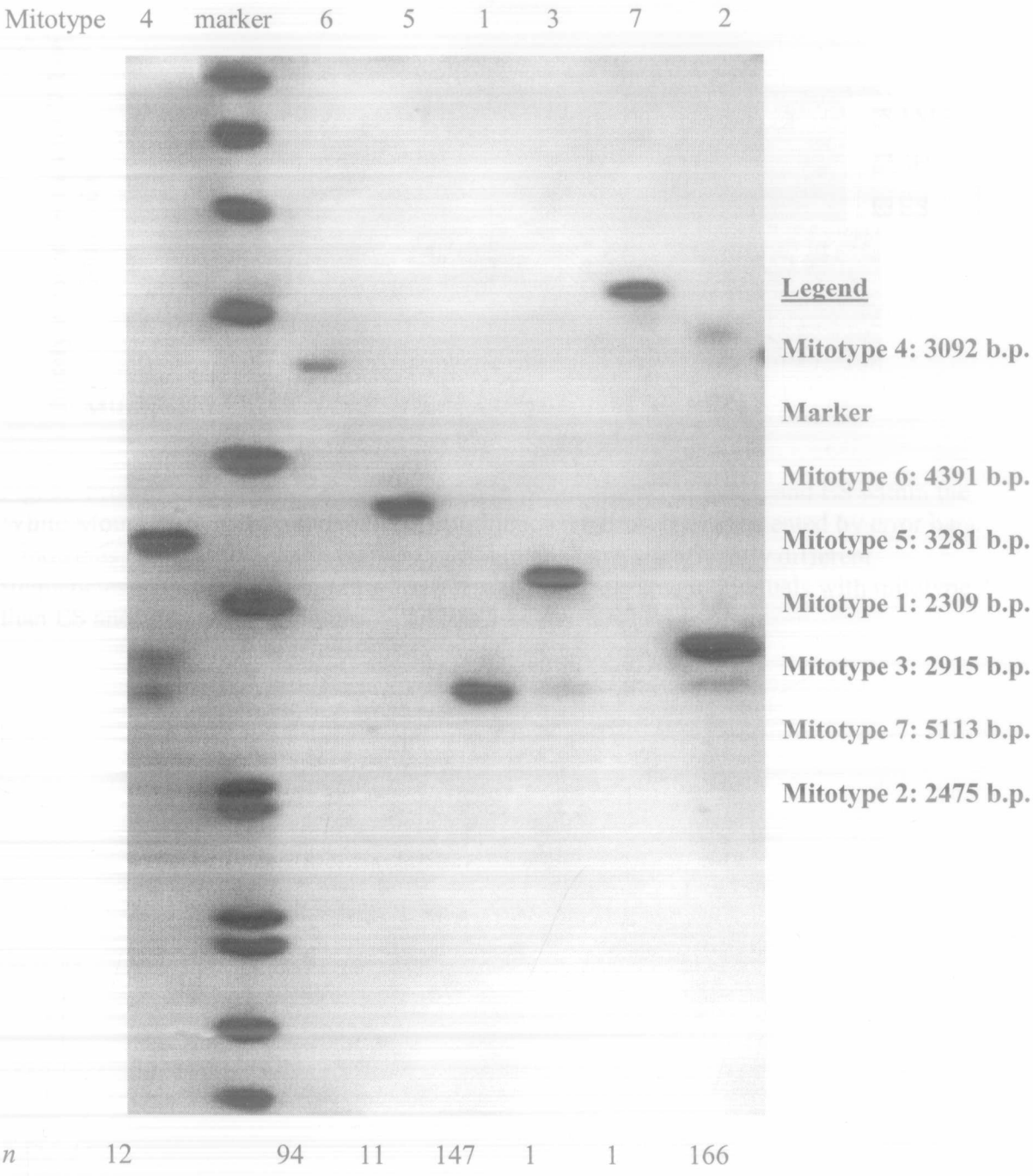

Fig. 4. Southern Blot showing mitochondrial *Hind III*-RFLP variation for the *COXI* target sequence among sites in the White Mountains; the Alaska Range; the Wrangell Mountains; and Norway. *n*= number of individuals with a mitotype.

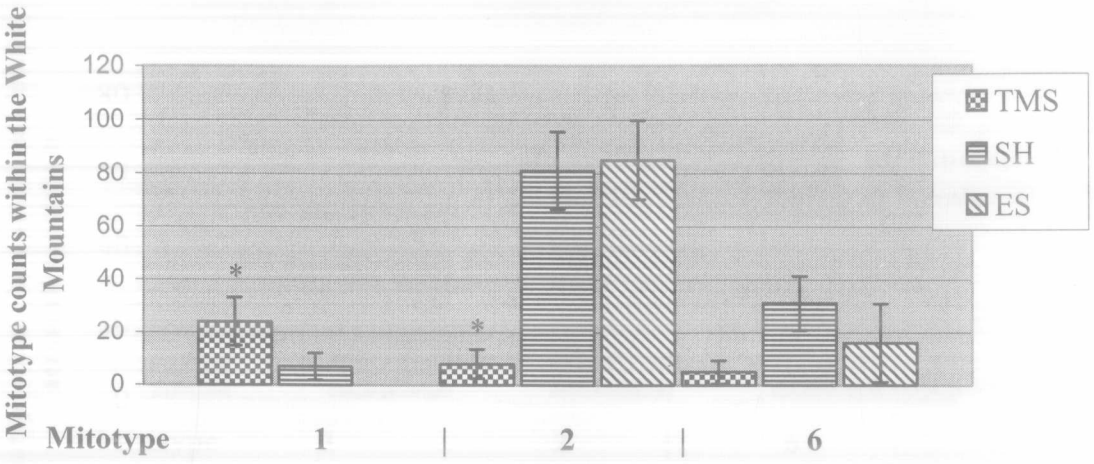

Fig. 5. Frequencies of mitotypes 1, 2, and 6 in populations TMS, SH, and ES within the White Mountains. Ninety-five percent confidence intervals are represented by error bars. * indicates that populations in the White Mountains have significantly different frequencies of mitotype 1, and that TMS has significantly less individuals with mitotype 2 than ES and SH.

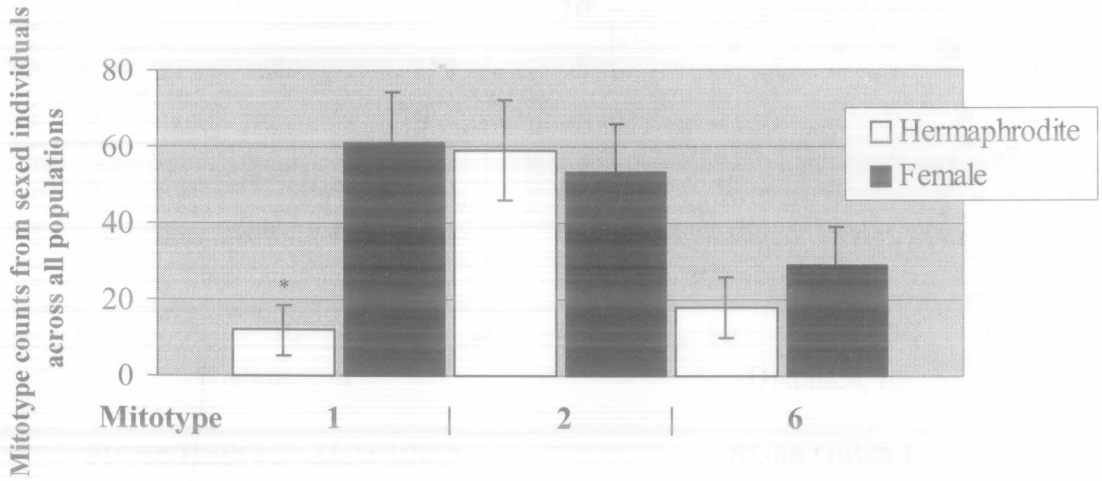

Fig. 6. Frequencies of mitotypes 1, 2, and 6 segregated by sex pooled across all populations. Ninety-five percent confidence intervals are represented by error bars. * indicates that the associations of hermaphrodites and females are different for mitotype 1.

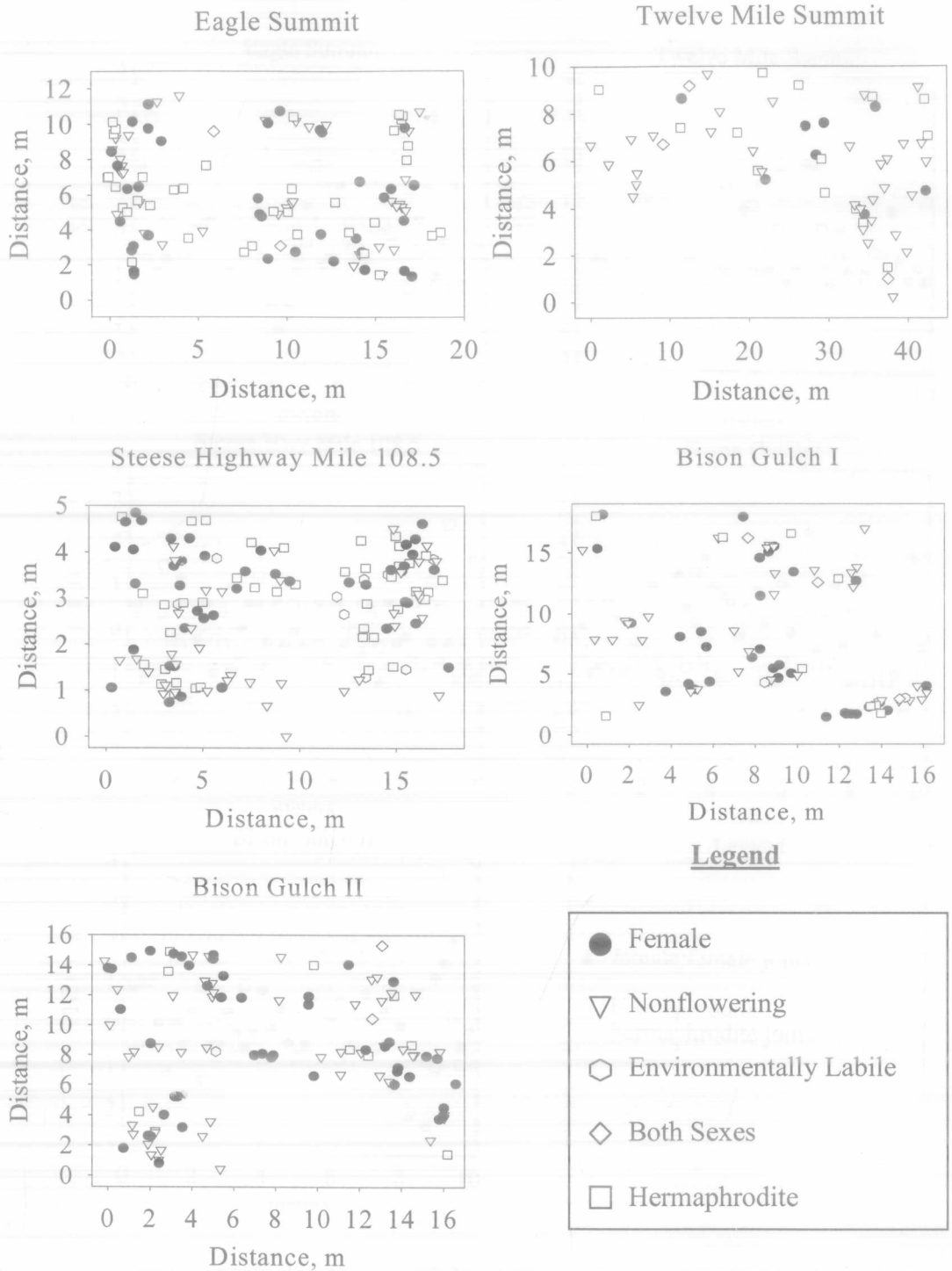

Fig. 7. Maps showing sexes of all individuals in Alaskan populations of *S. acaulis*.

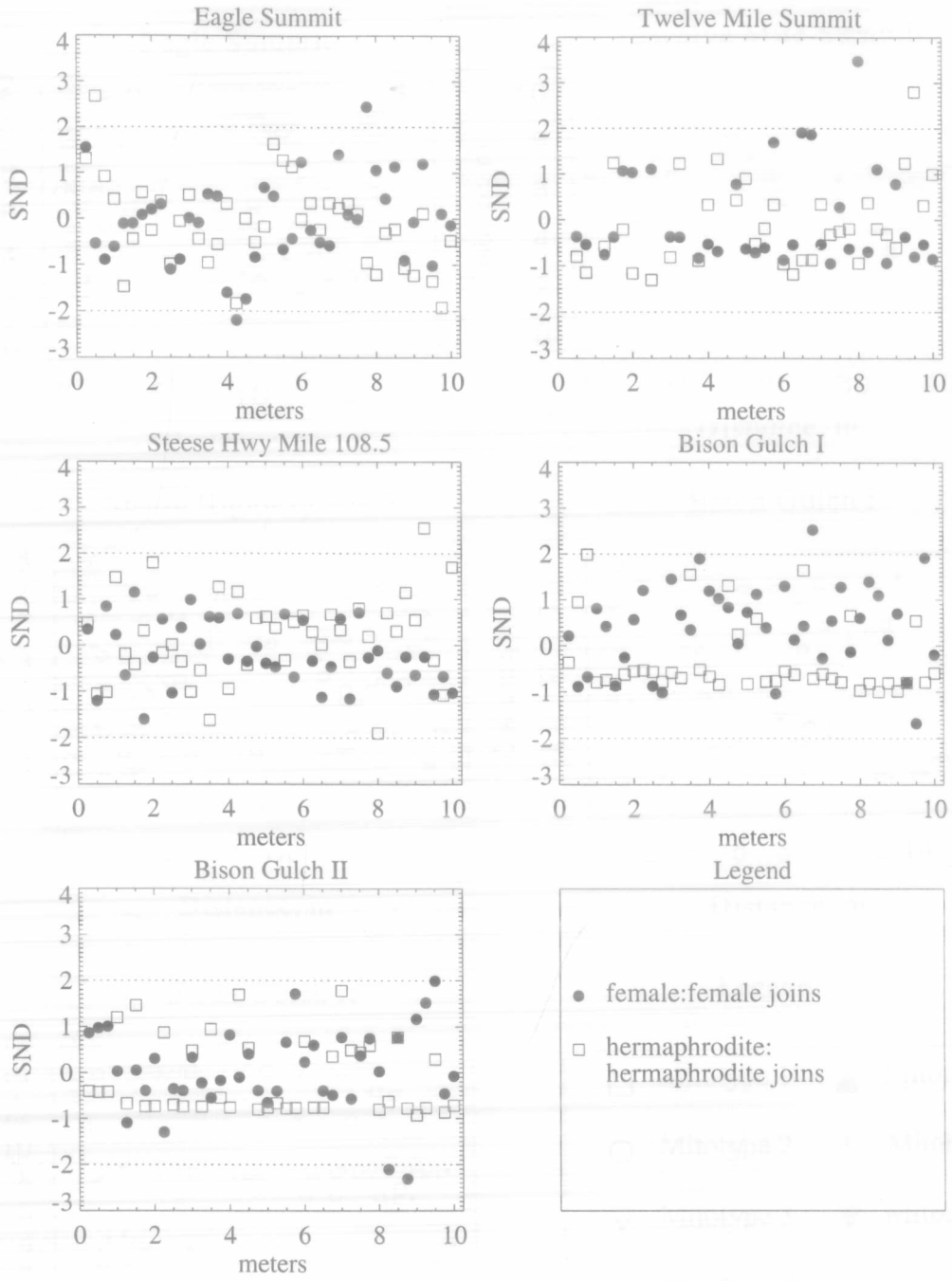

Fig. 8. Correllograms indicating spatial associations among individuals with the same sex in five Alaskan populations. Joins were tabulated at 0.25 m intervals. Standard Normal Deviate (SND) values (indicated by dotted lines) greater than 2 indicate clustering of individuals with a significance level of 95%. SND values less than -2 indicate repulsion of individuals.

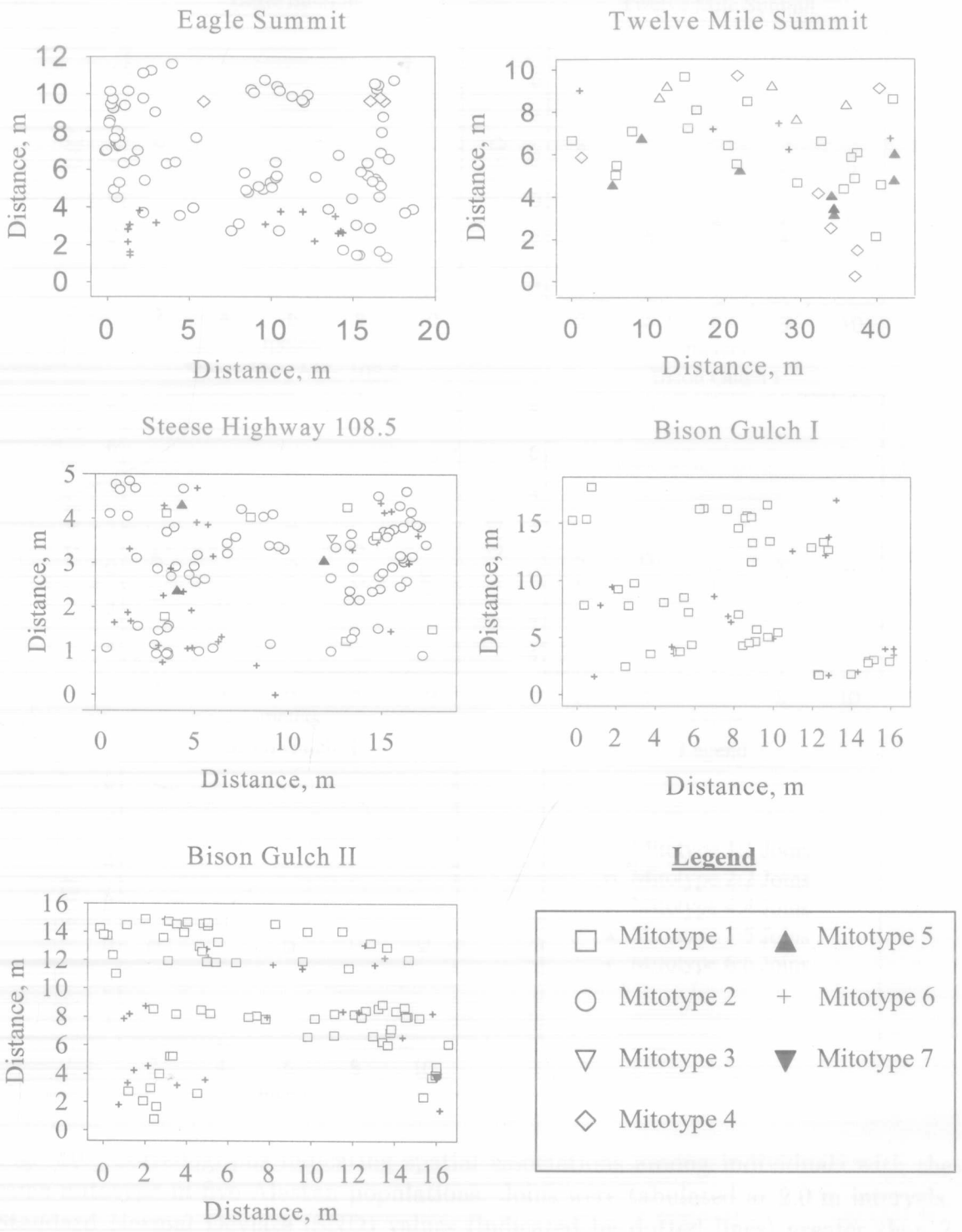

Fig. 9. Maps showing mitotypes of all individuals in Alaskan populations of *S. acaulis*.

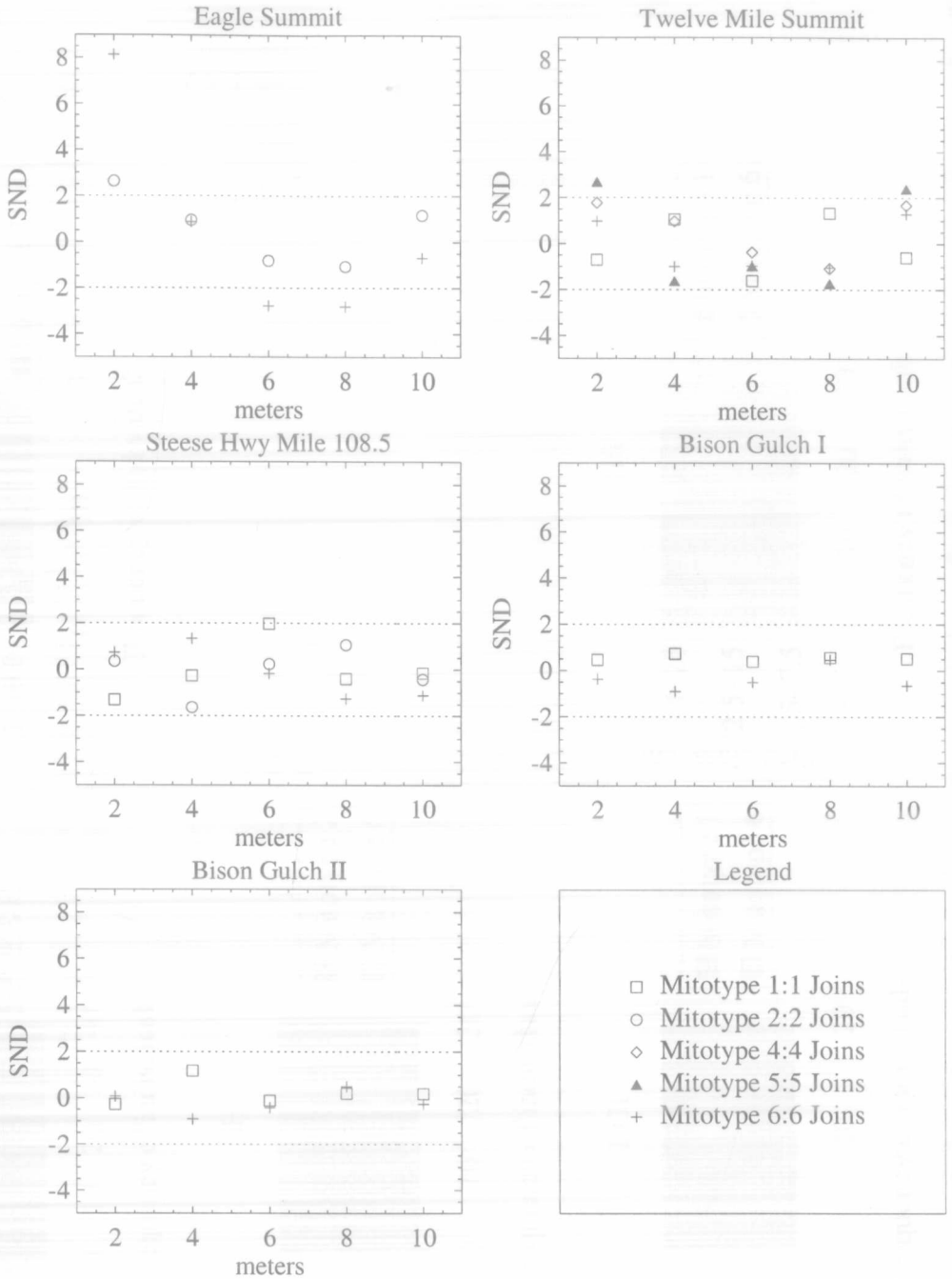

Fig. 10. Correllograms indicating spatial associations among individuals with the same mitotype in five Alaskan populations. Joins were tabulated at 2.0 m intervals. Standard Normal Deviate (SND) values (indicated by dotted lines) greater than 2 indicate clustering of individuals with a significance level of 95%. SND values less than -2 indicate repulsion of individuals.

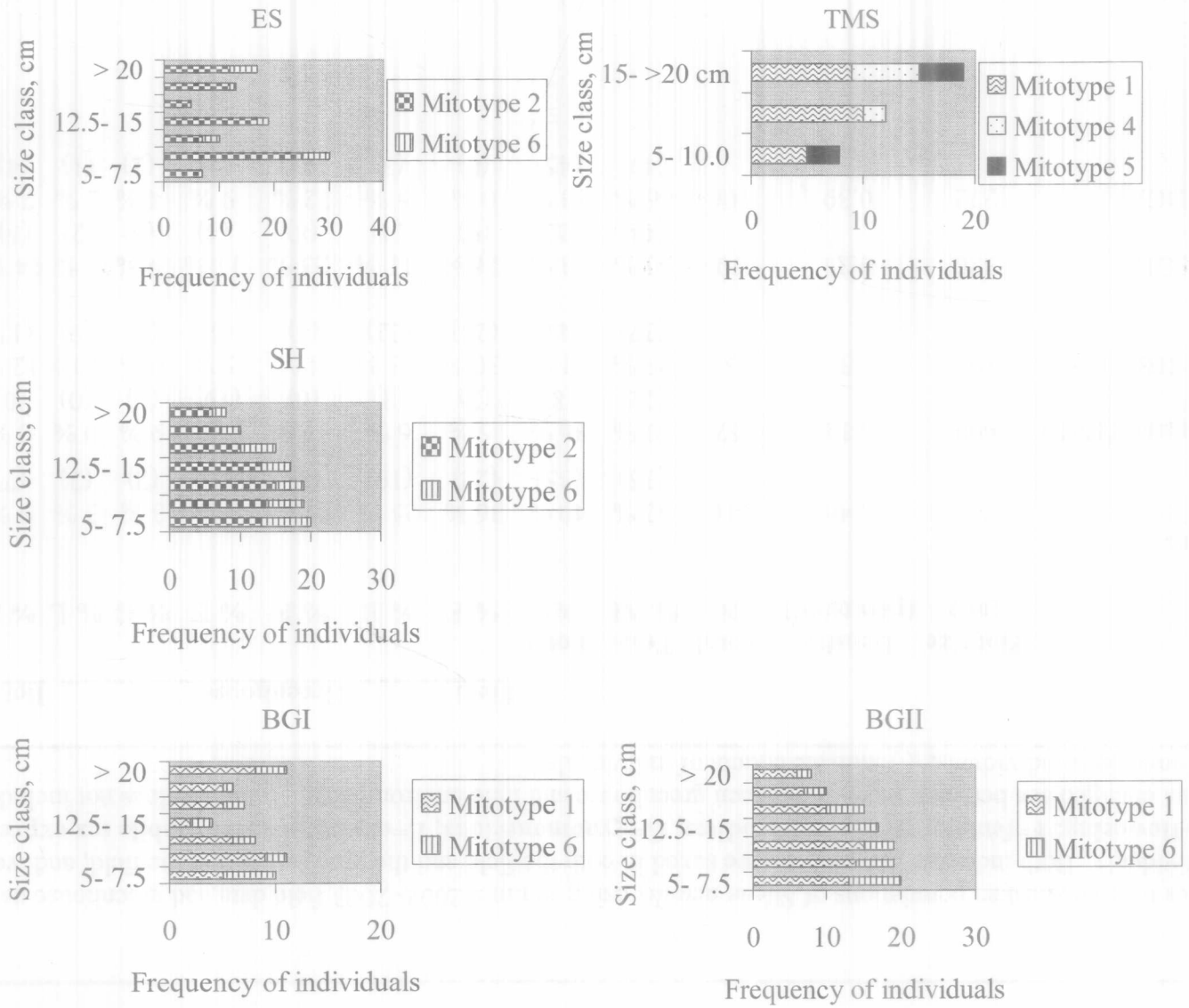

Fig. 11. Associations for size classes and mitotypes within populations. Size classes for all populations increase incrementally by 2.5 cm, beginning at 5cm. TMS had fewer individuals and so size classes were: 5- 10 cm, 10-15 cm, and 15-> 20 cm.

Table 1. Statistics for five Alaskan populations of *Silene acaulis* using summer 2002- 2003 field data and greenhouse data of transplanted individuals. Both indicates that plants were sexed in both the field and the greenhouse and the field, and were the same. **NF=non-flowering; F=female; H=hermaphrodites; G=gynomonecious; L=environmentally labile sex expression**, or individuals that changed sex between years or between greenhouse and field environments. Total %F does not include labile and gynomonecious individuals. ( ) indicate counts of individuals.

						Field		Greenhouse				Both	
Population	**Plot size (m^2)**	**Density (plants/m^2)**	**Total N**	**Total % NF**	**Total %F**	**% F**	**% H**	**% F**	**% H**	**% G**	**% L**	**% F**	**% H**
WhiteMountains													
Eagle Summit (ES)	240	0.46	110	32 % (35)	49 % (35)	36 % (27)	25 % (19)	1 % (1)	1 % (1)	3 % (2)	3 % (2)	9 % (7)	21 % (16)
Twelve Mile Summit (TMS)	400	0.14	57	39 % (22)	36 % (8)	6 % (2)	6 % (2)	17 % (6)	54 % (19)	9 % (3)	0 % (0)	0 % (0)	9 % (3)
Steese Highway 108.5 (SH)	100	1.31	131	28 % (37)	51 % (43)	30 % (28)	23 % (22)	4 % (4)	5 % (5)	0 % (0)	10 % (9)	12 % (11)	16 % (15)
Alaska Range													
Bison Gulch I (BGI)	320	0.25	79	56 % (44)	73 % (22)	26 % (9)	11 % (4)	23 % (8)	11 % (4)	9 % (3)	6 % (2)	14 % (5)	0 % (0)
Bison Gulch II (BGII)	272	0.39	106	46 % (49)	83 % (45)	0 % (0)	5 % (3)	72 % (41)	9 % (5)	4 % (2)	2 % (1)	7 % (4)	2 % (1)

Table 2. DNA sequences differences for three chlorotypes found in populations from the White Mountains, AK; the Alaska Range; the Wrangell Mountains; and Norway. Numbering of the consensus nucleotide position is indicated above the sequences and begins at the end of the *trnH* primer. *n* = number of individuals with a chlorotype.

Chlorotype	60 bp	74 bp	*n*
C	TAAAAAAAAGAAAAGAAAAAATTAAAAAAAAAGAAAAGAAAAAAA GAAAAAG		4
A	TA:::::::::::::::::::::AAAAAAAAGAAAAGAAAAAAA GAAAAAG		262
B	T:::::::::::::::::::::::::::::::::::::AAAAAAA GAAAAAG		170

Table 3. Population genetic differentiation estimates (θ, H_e) using RFLP mitotypes from the flanking region of the *COXI* locus.

	θ	H_e
Across populations	0.42	0.69
Across mountain ranges	0.42	
Across populations within mountain ranges		
White Mountains	0.30	0.58
Alaska Range	0.00	0.40

Table 4. Nei's genetic diversity (H_e) for the flanking region of the *COXI* mitochondrial locus by population. The number of haplotypes for the *COXI* locus is represented by A, and n is the sample size.

Mountain Range	Population	H_e	n
White Mountains	ES	0.32	105
	TMS	0.65	45
	SH	0.50	123
Alaska Range	BGI	0.42	63
	BGII	0.38	96

Table 5. Pairwise population genetic differentiation estimates (θ) using RFLP mitotypes from the *COXI* locus. Significance of θ values between mountain ranges was tested by Mantel's *r*.

	TMS	**ES**	**SH**	**BGI**	**BGII**
TMS	0				
ES	0.77	0			
SH	0.51	0.04	0		
BGI	0.10	0.95	0.63	0	
BGII	0.12	1.00	0.71	0	0

Table 6. Counts of individuals with each mitotype and chlorotype in Alaskan study populations.

	Mitotype							
	1	**2**	**3**	**4**	**5**	**6**	**7**	
Chlorotype								
B	116	0	0	0	0	42	1	**159**
A	28	116	1	11	11	52	0	**219**
C	3	0	0	1	0	0	0	**4**
Totals	**147**	**116**	**1**	**12**	**11**	**94**	**1**	

Table 7. Variable sites in the *Apocytochrome b* sequences from this study. S-AK-3 and S-AK-2 corresponded to Städler and Delph's (2002) haplotypes. Numbers at the right of the table (*n*) indicate the number of individuals with each *COXI*, *Apocytochrome b* combination that were observed in both the Alaska Range and White Mountains in this study.

Apocytochrome b	nucleotide position					*n*	
Mitotype	510	593	858	981	**CoxI mitotype**	AK	WM
S-AK-2	A	C	T	T	6	1	1
S-AK-3	A	A	A	G	1	2	2
SHIFT-6	C	C	A	T	6	-	1

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Appendix

```
PRO getdata,filename,sex,x,y
;=============================================================================
=
;NAME: getdata.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: gets parameters from a file and saves it into sex, x, and y
;PARAMETERS
;  filename:string containing complete path to file desired
;  sex:output array of integers
;  x: output array of floats
;  y: output array of floats
;NOTE: Format of file must be exact
;=====================================
a=intarr(300)
b=fltarr(300)
c=fltarr(300)

Openr,1,filename
i=0
WHILE(NOT EOF(1))DO BEGIN
        ReadF,1,FORMAT='(I1,1x,F6.3,11x,F6.3)',atmp,btmp,ctmp
        a[i]=atmp
        b[i]=btmp
    c[i]=ctmp
    i=i+1
ENDWHILE
CLOSE,1
sex=a[0:i-1]
x=b[0:i-1]
y=c[0:i-1]
END
```

```
Function getweight,r,D
;======================
;NAME: getweight.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: For all plant pairings, at each distance class sets the
;weight to zero if the interplant distance is greater than the
;distance class, otherwise the weight is 1.  Used by Joincount.pro.
;PARAMETERS
; D: gives distance from plant i to j, is 2D array of interplant distances
; r: sets range limit.  Array of distance classes.
;OUTPUT: 2D array of weights.
;======================
S=size(D)
N=S[1]
w=intarr(N,N)

For i=0,N-1 DO BEGIN
   w[i,i]=1 ;trivial case
   For j=i+1,N-1 DO BEGIN
      IF D[i,j] LE r THEN w[i,j]=1 ELSE w[i,j]=0
      w[j,i]=w[i,j]
   ENDFOR
ENDFOR
RETURN,w
END
```

```
FUNCTION joincount,sex,x,y,R,D
;=========================
;NAME: joincount.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY:
;performs a joincount where arrays of binary plant sex (female=1,
;hermaphrodite=0), and the corresponding x & y coords are input.
;R is an array of the distance classes to consider
;Outputs J=[M,3] where M is the number of distance classes, [M,0]=
;Female:Female [M,1]=Herm:Herm; [M,2]=Female:Herm
;PARAMETERS
;sex: array of integers corresponding to sex. e.g. Female=1, hermaphrodite=0.
;x: array of floats corresponding to x coordinates.
;y: array of floats corresponding to y coordinates.
;R: array of distance classes to examine.
;D: OPTIONAL, used by joinpermutation.pro to save time. A 2D array of
;all interplant distances.
;OUTPUT:
; 2D array of type int. 3 rows correspond to join types FF, HH, FH
; Columns correspond to the distance classes input in R.
;NOTE: Join counts are conducted on a DISK, so the largest distance
;class will include all earlier joins PLUS the additional joins in
;the additional distance. To get the join counts only in the
;additional distance, subtract the number of joins in the
;preceeding distance class.
;=================================

N=n_elements(sex)
M=n_elements(R)
IF n_elements(D) NE N*N  THEN D=plantdist(x,y)  ;call plantdist to
calculate all interplant distances, otherwise use passed values
Join=intarr(M,3)

FOR i=0,M-1 DO BEGIN
    FF=0
    HH=0
    FH=0
    w=getweight(R[i],D)
    FOR j=0,N-1 DO BEGIN
        FOR k=j+1,N-1 DO BEGIN
            CASE 1 OF
                sex[j] EQ 1 AND sex[k] EQ 1 :FF=FF+w[j,k]
                sex[j] EQ 0 AND sex[k] EQ 0 :HH=HH+w[j,k]
                ELSE: FH=FH+w[j,k]
            ENDCASE
        ENDFOR
    ENDFOR
```

```
    Join[i,0]=FF
    Join[i,1]=HH
    Join[i,2]=FH
ENDFOR
RETURN,Join
END
```

```
FUNCTION joincount2,sex,x,y,R,D
;=========================
;NAME: joincount2.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: performs a joincount where arrays of plant mitotype (1-6)
;and the corresponding x & y coords are input.
;R is an array of the distance classes to consider
;Outputs J=[M,7] where M is the number of distance classes, [M,0]=
;crosscounts [M,1]=1:1; [M,2]=2:2,etc...
;PARAMETERS
;sex: array of mitotypes (1-6)
;x: array of floats corresponding to x coordinates.
;y: array of floats corresponding to y coordinates.
;R: array of distance classes to examine.
;D: OPTIONAL, used by joinpermutation2.pro to save time.  A 2D array of
;all interplant distances.
;OUTPUT:
; 2D array of type int.  7 rows correspond to joins of similiar
; mitotype. Oth row is all cross joins (e.g. 1:2, 2:5, etc.).
; Columns correspond to the distance classes input in R.
;NOTE: Join counts are conducted on a DISK, so the largest distance
;class will include all earlier joins PLUS the additional joins in
;the additional distance.  To get the join counts only in the
;additional distance, subtract the number of joins in the
;preceeding distance class.
;=================================

N=n_elements(sex)
M=n_elements(R)
IF n_elements(D) NE N*N  THEN D=plantdist(x,y)  ;call plantdist to
calculate all interplant distances, otherwise use passed values
Join=intarr(M,7)

FOR i=0,M-1 DO BEGIN
    oo=0 ;one
    tt=0 ;Set all joins to zero
    thth=0 ;3
    ff=0    ;4
    fifi=0  ;5
    ss=0    ;6
    xx=0  ;cross joins
    w=getweight(R[i],D) ;not problem
    FOR j=0,N-1 DO BEGIN
        FOR k=j+1,N-1 DO BEGIN
            IF sex[j] EQ sex[k] THEN BEGIN  ;same mitotype
                CASE 1 OF
                    sex[j] EQ 1 :oo=oo+w[j,k]
```

```
                    sex[j] EQ 2 :tt=tt+w[j,k]
                    sex[j] EQ 3 :thth=thth+w[j,k]
                    sex[j] EQ 4 :ff=ff+w[j,k]
                    sex[j] EQ 5 :fifi=fifi+w[j,k]
                    sex[j] EQ 6 :ss=ss+w[j,k]
                ENDCASE
            ENDIF ELSE xx=xx+w[j,k]
        ENDFOR
    ENDFOR
    Join[i,0]=xx
    Join[i,1]=oo
    Join[i,2]=tt
    Join[i,3]=thth
    Join[i,4]=ff
    Join[i,5]=fifi
    Join[i,6]=ss
ENDFOR
RETURN,Join
END
```

```
FUNCTION joindeviate,Join,joinperm
;=====================================
;NAME: joindeviate.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: Calculates the z-normal deviate input a joincount.pro output: JOIN
;and a joinpermuation.pro output: JOINPERM
;output[N,3], Where N is the number of distance classes. [*,0]=FF
;[*,1]=HH,[*,2]=FH.  gives (x-<x>)/Sqrt(Var(<x>))
;PARAMETERS
;Join: output of joincount of data
;joinperm: output from joinpermutation.pro
;OUTPUT: 2D array which contains the standard deviation of each join
;type at each distance class.
;=====================================
s=size(joinperm)
M=s[1]  ;number of random joinpermutations
N=s[2]  ;number of distance classes
output=fltarr(N,3)

FOR i=0,N-1 DO BEGIN ;for each distance class
   momff=moment(joinperm[*,i,0])
   momhh=moment(joinperm[*,i,1])
   momfh=moment(joinperm[*,i,2])
   output[i,0]=(join[i,0]-momff[0])/sqrt(momff[1])
   output[i,1]=(join[i,1]-momhh[0])/sqrt(momhh[1])
   output[i,2]=(join[i,2]-momfh[0])/sqrt(momfh[1])
ENDFOR
RETURN,output
END
```

```
FUNCTION joindeviate2,Join,joinperm
;=====================================
;NAME: joindeviate2.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: Calculates the z-normal deviate input a joincount2.pro output: JOIN
;and a joinpermuation2.pro output: JOINPERM
;output[N,3], Where N is the number of distance classes. [*,0]=FF
;[*,1]=HH,[*,2]=FH.  gives (x-<x>)/Sqrt(Var(<x>))
;PARAMETERS
;Join: output of joincount2 of data
;joinperm: output from joinpermutation2.pro
;OUTPUT: 2D array which contains the standard deviation of each join
;type at each distance class.
;=====================================
s=size(joinperm)
M=s[1]  ;number of random joinpermutations
N=s[2]  ;number of distance classes
output=fltarr(N,7)

FOR i=0,N-1 DO BEGIN ;for each distance class
   momxx=moment(joinperm[*,i,0])
   momoo=moment(joinperm[*,i,1])
   momtt=moment(joinperm[*,i,2])
   momthth=moment(joinperm[*,i,3])
   momff=moment(joinperm[*,i,4])
   momfifi=moment(joinperm[*,i,5])
   momss=moment(joinperm[*,i,6])
   output[i,0]=(join[i,0]-momxx[0])/sqrt(momxx[1])
   output[i,1]=(join[i,1]-momoo[0])/sqrt(momoo[1])
   output[i,2]=(join[i,2]-momtt[0])/sqrt(momtt[1])
   output[i,3]=(join[i,3]-momthth[0])/sqrt(momthth[1])
   output[i,4]=(join[i,4]-momff[0])/sqrt(momff[1])
   output[i,5]=(join[i,5]-momfifi[0])/sqrt(momfifi[1])
   output[i,6]=(join[i,6]-momss[0])/sqrt(momss[1])
ENDFOR
RETURN,output
END
```

```
FUNCTION joinpermutation,P,sex,x,y,r
;================================
;NAME: joinpermutation.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: Does P joincounts, permutating the sex array each time for
;statistical purposes output is 3D array Consisting of the F:F, H:H,
;and F:H count for each r value for each permutation of the sex array.
;PARAMETERS
;P: number of permutations to perform.
;sex: array of binary sex classes
;x: array of x coordinates
;y: array of y coordinates
;r: array of distance classes
;OUTPUT: 3D array.  Dim-1 is the permutation number. Dim-2 is the
;distance class, and Dim-3 is the join type.
;NOTE: For Large data sets and P, this may take a very long time.
;============================
M=n_elements(r)
output=intarr(P,M,3)
D=plantdist(x,y) ;so joincount doesn't have to calculate on each iteration.
FOR i=0,P-1 DO BEGIN
        sexc=shuffle(sex)
        output[i,*,*]=joincount(sexc,x,y,r,D)
ENDFOR
RETURN,output
END
```

```
FUNCTION joinpermutation2,P,sex,x,y,r
;================================
;NAME: joinpermutation2.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: Does P joincounts, permutating the sex array each time for
;statistical purposes output is 3D array Consisting of the F:F, H:H,
;and F:H count for each r value for each permutation of the sex array.
;PARAMETERS
;P: number of permutations to perform.
;sex: array of mitotype classes
;x: array of x coordinates
;y: array of y coordinates
;r: array of distance classes
;OUTPUT: 3D array.  Dim-1 is the permutation number. Dim-2 is the
;distance class, and Dim-3 is the join type.
;NOTE: For Large data sets and P, this may take a very long time.
;============================
M=n_elements(r)
output=intarr(P,M,7)
D=plantdist(x,y) ;so joincount doesn't have to calculate on each iteration.
FOR i=0,P-1 DO BEGIN
        sexc=shuffle(sex)
        output[i,*,*]=joincount2(sexc,x,y,r,D)
ENDFOR
RETURN,output
END
```

```
FUNCTION plantdist,x,y
;=======================
;NAME: plantdist.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY:  given arrays of x,y coords, returns matix of D[i,j] consisting of
;distance from plant i to plant j
;PARAMETERS
;x: array of floats
;y: array of floats
;OUTPUT: 2D array that is the size of x in both dimensions.  Element
;[i,j] is the linear distance from plant i to plant j.
;=========================

N=n_elements(x)
Dist=fltarr(N,N)
FOR i=0,N-1 DO BEGIN
   FOR j=i+1,N-1 DO BEGIN
      Dist[i,j]=Sqrt((x[i]-x[j])^2 +(y[i]-y[j])^2)
      Dist[j,i]=Dist[i,j] ;Probably not needed, but just in case.
   ENDFOR
ENDFOR
RETURN,Dist
END
```

```
Function rejoin,data
;=======================
;does joincount only counting joins in current distance class,
;excluding smaller distance classes, thus the regions form an annulus
;input computed joincount, or joinpermutation output.
;=======================

N=size(data)
IF N[0] EQ 2 THEN BEGIN ;assume form j[M,*] joincount output
   output=intarr(N[1],N[2])
   output[0,*]=data[0,*]
   FOR i=1,n[1]-1 DO output[i,*]=data[i,*]-data[i-1,*] ;remove joincount of previous
distance class
ENDIF ELSE BEGIN
   IF N[0] EQ 3 THEN BEGIN
      output=intarr(N[1],N[2],N[3])
      output[*,0,*]=data[*,0,*]
      FOR i=1,n[2]-1 DO output[*,i,*]=data[*,i,*]-data[*,i-1,*]
   ENDIF ELSE print,'Invalid input'
ENDELSE
RETURN,output
END
```

```
FUNCTION shuffle,input
;=====================
;NAME: shuffle.pro
;WRITTEN BY: Jon Klaas, jpklaas@yahoo.com
;DATE: 6/14/2004
;SUMMARY: reorders input array randomly
;PARAMETERS
;input: array
;OUTPUT: reordered input array.
;=====================
N=n_elements(input)
output=intarr(N)
inputc=input ;avoid altering input. Pass by reference sucks sometimes.
COMMON block1, seed ;contains random number seed for future shuffles to avoid same
shuffle
FOR i=0,N-1 DO BEGIN
   M=n_elements(inputc)
   j=FLOOR(M*randomu(seed));choose next element of output
   output[i]=inputc[j]
   ;resize input
        CASE 1 of
                M EQ 1:BREAK ;loop is done
                j EQ M-1:inputc=inputc[0:j-1] ;j is largest value
                j EQ 0:inputc=inputc[1:M-1] ;j is smallest value
                ELSE: inputc=[inputc[0:j-1],inputc[j+1:M-1]] ;compresses inputc
        ENDCASE
ENDFOR
RETURN,output
END
```